Interactions and Metabolic Potential of Groundwater Microorganisms

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Science
makes people reach selflessly for truth and objectivity;
it teaches people to accept reality
with wonder and admiration

- Lise Meitner

Summary

In oligotrophic environments like groundwater, microorganisms can adapt to the nutrient limited conditions through the development of cooperative or antagonistic interactions, or through the general adaptation and regulation of their metabolism. Microbial communities in groundwater are highly complex and often dominated by microorganisms that lack cultivated representatives, including members of the Candidate Phyla Radiation (CPR). These ultra-small sized bacteria have streamlined genomes that lack metabolic functions thought to be essential for life, suggesting that they depend on other microorganisms for these cellular components. This lifestyle hampers the cultivation of CPR, which traditionally relies upon the isolation of single microorganisms. Thus, understanding the repertoire of interaction mechanisms and the metabolic potential of groundwater bacteria is limited to laboratory investigations of a few isolates or based exclusively on genome-derived information.

To study these critical and understudied inter- and intra-species interactions, the production of growth promoting- and inhibiting metabolites by groundwater bacteria was evaluated. To do so, I developed a high-throughput screening method that allowed a rapid and highly standardized evaluation in liquid cultures. This method enabled the screening of 149 bacterial isolates from groundwater and 1402 co-cultures, revealing a high potential for the production of growth-affecting secondary metabolites. My results showed that 38 % of the tested bacteria had growth inhibitory effects and 6 % caused growth promotion when grown in monoculture, but both of these effects were reduced by 50 % when bacteria were grown in co-cultures. Although cooperative interactions were expected to be dominating, antagonistic interactions were more prevalent among these cultivated representatives of the groundwater community.

To target interactions between uncultivated microorganisms and identify the potential partners of the CPR, co-occurrence network analyses were performed. CPR were found to play a central role in the microbial community and are associated with specific partner organisms, including abundant autotrophic bacteria. To determine the underlying mechanisms behind these predicted interactions, I performed a detailed analysis of one CPR: *Cand.* Roizmanbacterium ADI133 possesses a typical CPR genome which lacks the genetic potential to synthesize certain amino acids or lipids. Within the genome, I identified potential genetic machinery that would enable this bacterium to attach to its necessary partner organisms. The subsequent development of a specific primer set allowed me to track *Cand.* Roizmanbacterium ADI133, and I showed that it comprises up to 2.3 % of the bacterial community. A complete genomic characterization and co-occurrence analysis pointed to a host-dependent lifestyle in which the Roizmanbacterium

provides lactate to its mixotrophic partner bacteria. This transfer of organic carbon is thought to stimulate the growth of other community members with important ramification for sulfur and nitrogen cycling. Thus, these results imply that cooperative interactions within the yet uncultivated portion of the groundwater microbiome play a more pronounced role than suggested by cultivation-based findings.

In addition to the high abundances of members of the CPR, hetero- and autotrophic bacteria were surprisingly found in the ultra-small fraction (< 0.2 µm) of bacteria in groundwater. On a subset of representative heterotrophic bacteria isolated from the site, I was able to show that the reduction of carbon supply leads to a decrease of cell size of 80 %, matching our field observations. These findings suggest that groundwater microorganisms are adjusted to carbon starvation in the environment by increasing their surface-to-volume ratio to optimize the uptake of sparse nutrients.

As the input of surface-derived organic carbon is limited, dead microbial biomass (necromass) might be recycled by the groundwater microbiome. When I followed the uptake of necromass-derived carbon using a combined DNA- and Protein Stable Isotope Probing (SIP) approach, heterotrophic members of the community rapidly responded and stimulated the growth of autotrophic organisms. Notably, members of the CPR did not incorporate necromass-derived carbon even though necromass is a source of the amino acids, lipids and vitamins that cannot be produced by CPR. Furthermore, our data show that eukaryotic members of the groundwater microbiome incorporate necromass-derived carbon either by directly feeding on necromass or on the biomass of the heterotrophic bacteria. In addition, we show that more complex food webs coincide with a higher genetic potential for chemolithoautotrophic primary production suggesting that complex food webs in groundwater are supported by chemolithoautotrophy as well as an efficient recycling of necromass.

Collectively, the findings presented within this dissertation demonstrate how groundwater microbial communities are structured and influenced by species interactions as well as by the metabolic potential of individual members. While the predicted role of key players like *Cand.* Roizmanbacterium ADI133 has significantly contributed to the understanding of groundwater ecology, this dissertation further highlights the importance of trophic interactions in the terrestrial subsurface. However, novel cultivation strategies are needed to learn more about the role of yet uncultivated microorganisms, including the CPR.

Zusammenfassung

In oligotrophen Lebensräumen wie Grundwasser können sich Mikroorganismen durch die Entwicklung kooperativer oder antagonistischer Interaktionen oder durch die allgemeine Anpassung und Regulierung ihres Stoffwechsels an die nährstoffarmen Bedingungen anpassen. Mikrobielle Gemeinschaften im Grundwasser sind hochkomplex und werden oft von Mikroben dominiert, denen es an kultivierten Vertretern mangelt, darunter Bakterien der Candidate Phyla Radiation (CPR). Diese sehr kleinen Bakterien haben reduzierte Genome, denen essenzielle Stoffwechselfunktionen fehlen, was darauf hindeutet, dass sie von anderen Mikroorganismen abhängig sind. Dieser Lebensstil erschwert die Kultivierung von CPR, welche traditionell auf der Isolierung einzelner Mikroorganismen beruht. Das Verständnis des Repertoires der Interaktionsmechanismen und des metabolischen Potenzials von Grundwasserbakterien beschränkt sich daher auf Laboruntersuchungen einiger weniger Isolate oder basiert ausschließlich auf genomischen Informationen.

Um die wenig erforschten Interaktionen zwischen und innerhalb einer Spezies zu untersuchen, wurde die Produktion von wachstumsfördernden und hemmenden Metaboliten durch Grundwasserbakterien untersucht. Dazu wurde von mir ein Hochdurchsatz-Screeningverfahren entwickelt, welches eine schnelle und hochstandardisierte Auswertung in Flüssigkulturen ermöglicht. Dieses Verfahren ermöglichte das Screening von 149 Bakterienisolaten aus Grundwasser und 1402 Co-Kulturen welche ein hohes Potenzial für die Produktion von wachstumsbeeinflussenden Sekundärmetaboliten aufweisen. Meine Ergebnisse zeigten, dass 38 % der getesteten Bakterien wachstumshemmende und 6 % wachstumsfördernde Effekte verursachten, wenn sie in Monokulturen gezüchtet wurden. Beide Effekte wurden um 50 % reduziert, wenn Bakterien in Co-Kulturen wuchsen. Obwohl erwartet wurde, dass kooperative Interaktionen dominieren, waren antagonistische Interaktionen unter diesen kultivierten Vertretern der Grundwassergemeinschaft stärker verbreitet.

Um Interaktionen zwischen nicht-kultivierten Mikroorganismen gezielt zu untersuchen und die potenziellen Partner der CPR zu identifizieren, wurden Co-Occurrence Netzwerkanalysen durchgeführt. Es wurde festgestellt, dass die CPR eine zentrale Rolle in der mikrobiellen Gemeinschaft spielen und mit spezifischen Partnerorganismen, einschließlich vorhandener autotropher Bakterien, assoziiert sind. Um die zugrunde liegenden Mechanismen hinter diesen vorhergesagten Interaktionen zu bestimmen, habe ich eine detaillierte Analyse eines CPR durchgeführt: *Cand.* Roizmanbacterium ADI133 besitzt ein typisches CPR-Genom, in dem das

genetische Potential einige Aminosäuren oder Lipide zu synthetisieren fehlt. Im Genom habe ich potenzielle genetische Mechanismen identifizieren können, welche es diesem Bakterium ermöglichen würden, sich an seine notwendigen Partnerorganismen anzuheften. Die anschließende Entwicklung eines speziellen Primersets ermöglichte es mir, *Cand.* Roizmanbacterium ADI133 zu quantifizieren und nachzuweisen, dass es bis zu 2,3 % der Bakteriengemeinschaft ausmacht. Eine vollständige genomische Charakterisierung und Co-Occurrenceanalyse deutete auf einen wirtsabhängigen Lebensstil hin, bei dem das Roizmanbacterium seinen mixotrophen Partnerbakterien Laktat zur Verfügung stellt. Dieser Transfer von organischem Kohlenstoff könnte das Wachstum anderer Mitglieder der Gemeinschaft anregen, mit wichtigen Auswirkungen für den Schwefel- und Stickstoffkreislauf. Diese Ergebnisse deuten somit darauf hin, dass kooperative Wechselwirkungen innerhalb des noch nicht kultivierten Teils des Grundwassermikrobioms eine ausgeprägtere Rolle spielen, als es kultivierungsbasierte Erkenntnisse vermuten lassen.

Neben den hohen Häufigkeiten von Mitgliedern der CPR wurden hetero- und autotrophe Bakterien überraschend in der kleinen Fraktion (< 0,2 µm) von Bakterien im Grundwasser gefunden. An heterotropen Bakterien, die aus dem Grundwasser isoliert wurden, konnte ich zeigen, dass die Reduzierung der Kohlenstoffversorgung zu einer Verringerung der Zellgröße um 80 % führt, was unseren Feldbeobachtungen entspricht. Diese Ergebnisse deuten darauf hin, dass Grundwassermikroorganismen an den Kohlenstoffmangel in der Umwelt angepasst sind, indem sie ihr Verhältnis von Oberfläche zu Volumen erhöhen, um die Aufnahme von begrenzten Nährstoffen zu optimieren.

Da der Eintrag von organischem Kohlenstoff begrenzt ist, kann tote mikrobielle Biomasse (Nekromasse) durch das Grundwassermikrobiom recycelt werden. In einer Untersuchung der Aufnahme von Nekromasse mittels kombinierten DNA- und Protein Stable Isotope Probings (SIP), reagierten heterotrophe Mitglieder der Gemeinschaft schnell und stimulierten das Wachstum autotropher Organismen. Insbesondere haben die Mitglieder der CPR jedoch keinen aus Nekromasse gewonnenen Kohlenstoff aufgenommen, obwohl Nekromasse eine Quelle für Aminosäuren, Lipide und Vitamine ist, die von CPR nicht selbst produziert werden können. Darüber hinaus zeigen unsere Daten, dass eukaryotische Mitglieder des Grundwassermikrobioms Nekromasse-Kohlenstoff entweder durch direkte Aufnahme von Nekromasse oder aus der Biomasse der heterotrophen Bakterien aufnehmen. Darüber hinaus zeigen diese Ergebnisse, dass komplexere Nahrungsnetze mit einem höheren genetischen Potenzial für chemolithoautotrophe Primärproduktion zusammenfallen, was darauf hindeutet,

dass Nahrungsnetze im Grundwasser durch Chemolithoautotrophie, sowie ein effizientes Recycling von Nekromasse unterstützt werden.

Zusammenfassend zeigen die in dieser Dissertation vorgestellten Ergebnisse, wie mikrobielle Grundwassergemeinschaften strukturiert sind und durch Interaktionen sowie durch das metabolische Potenzial einzelner Mitglieder beeinflusst werden. Neben der postulierten Rolle von Schlüsselorganismen wie *Cand.* Roizmanbacterium ADI133, welche wesentlich zum Verständnis der Grundwasserökologie beigetragen hat, unterstreicht diese Dissertation ebenfalls die Bedeutung trophischer Wechselwirkungen im terrestrischen Untergrund. Jedoch sind neue Kultivierungsstrategien erforderlich, um mehr über die Rolle der noch nicht kultivierten Mikroorganismen, einschließlich der CPR, zu erfahren.

Contents

1. Introduction

1.1 Microbial Interactions

Microorganisms are in constant interaction with their environment, but also with other organisms of the same (intraspecific interactions) or other species (interspecific interactions). Biological interactions between bacteria can have strong effects on the entire microbial community in terms of bacterial growth, community composition and functioning (Hibbing *et al.*, 2010; Pande and Kost, 2017). Interspecific interactions can be beneficial for both sides (mutualism), only for one side (commensalism) or beneficial for one side while the other organism is deprived (parasitism). While in the past "symbiosis" has often been defined as a positive-positive interaction, nowadays all persistent biological interactions of different organisms are defined as "symbiosis", regardless of the type of interaction (Martin and Schwab, 2012; **Figure 1**). Between microorganisms, these interactions are often mediated by the exchange of extracellular metabolites.

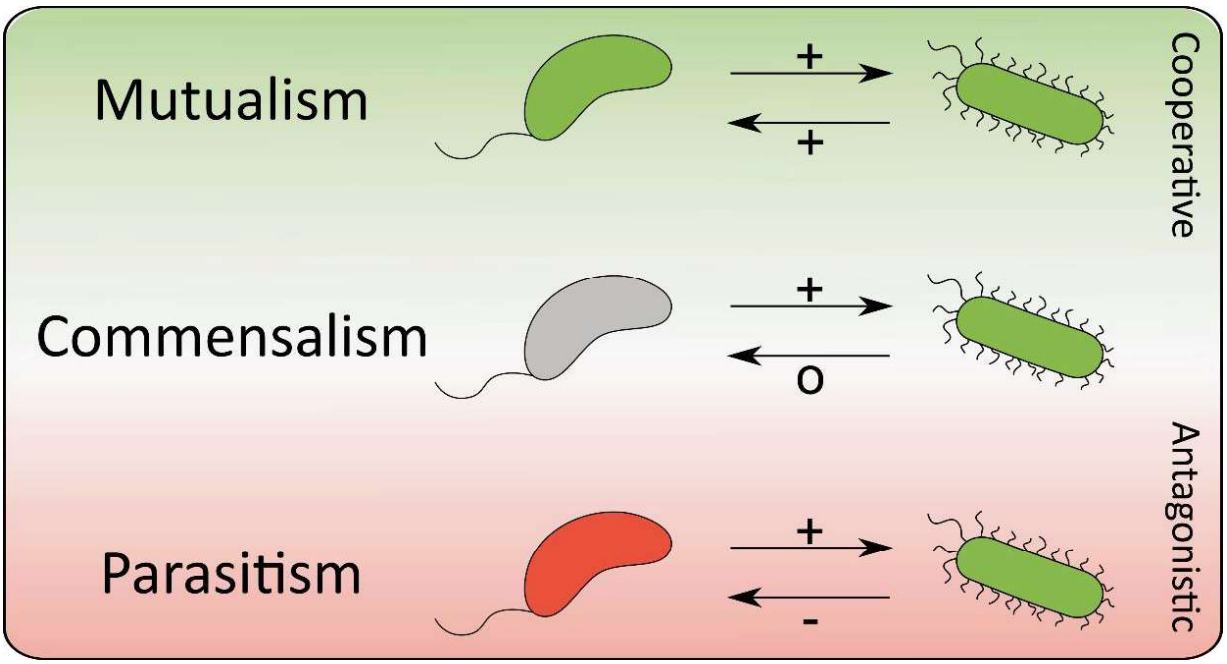

Figure 1 | Different forms of symbiotic interactions between microorganisms.
Mutualism, commensalism and parasitism can be categorized as cooperative and antagonistic interactions. Benefitting bacteria are depicted in green, whereas the deprived bacterium is depicted in red. Grey indicates a neutral interaction.

1.1.1 Interactions via Primary and Secondary Metabolites

Microorganisms continuously produce metabolites (Greek: *metabolítes*, "the changed") that can have various functions within the cell, such as determining cell structure, signalling or regulating enzymes. Generally, metabolites can be distinguished as primary and secondary metabolites, with primary metabolites resulting from basic cellular functions like growth or reproduction. They are crucial for the functioning and survival of a cell. Contrastingly, secondary metabolites are not central in a cell's metabolism; however, they are direct mediators of an organism with its environment and other organisms. Secondary metabolites produced by bacteria can be antimicrobial compounds but also growth factors, that can affect other microorganisms positively or negatively (Hibbing *et al.*, 2010; Cornforth and Foster, 2013; Pande and Kost, 2017; **Figure 1**).

Bacterial metabolites can directly impact other organisms or are used as signalling compounds for surrounding bacteria. In order to regulate processes like biofilm formation or antimicrobial substances production within a population of the same species, some bacteria use "quorum sensing" to determine population cell densities within their environment (Waters and Bassler, 2005). As soon as a critical density is reached these bacteria produce or stop producing metabolites such as antimicrobial compounds (Holden *et al.*, 1998; Waters and Bassler, 2005). In this example, an intraspecific interaction leading to a "quorum" among bacteria of one species, is followed by interspecific interactions via antibiotics with the microbial community.

Antagonism. Antagonistic interactions between microorganisms by the release of secondary metabolites like antibiotics, as well as parasitic interactions, are common in microbial communities (Foster and Bell, 2012; Pande and Kost, 2017). While some bacteria and micro-eukaryotes are well known for being parasites of higher organisms, bacteria can also act as parasites on other bacteria. For example members of the genus *Bdellovibrio*, which attack, enter and subsequently replicate inside the host cells (Stolp, 1973).

Likewise, the production of antibiotics negatively affects the growth of other microorganisms that are in direct competition for e.g. nutrients or space within an environment, and thereby promotes the growth of the producing bacterium. This costly production of secondary metabolites, however, has been shown not be continuously performed but rather to be encoded on gene clusters that are only actively expressed when the bacterium is triggered by surrounding bacteria (Scherlach and Hertweck, 2009; Cornforth and Foster, 2013). Experimental studies confirm that secondary metabolites are regularly a result of direct interaction with other bacteria

(Garbeva *et al.*, 2011; Traxler *et al.*, 2013; Tyc *et al.*, 2014, 2017) and that the production of antimicrobial compounds increases when bacteria are grown in pairwise interactions (Kinkel *et al.*, 2014). Not only in environments with higher cell numbers, but also in more dilute, aquatic ecosystems, the production of antimicrobial substances against other bacteria plays a role (Motta *et al.*, 2004). In fact, especially less studied, extreme environments like the deep-sea are promising sources of microorganisms that produce compounds with antimicrobial activity (reviewed in Tortorella *et al.*, 2018). Similarly, microbial communities in the terrestrial subsurface can hold the metabolic potential of interacting with other microorganisms via antimicrobial compounds.

Cooperation. In cooperative interactions multiple organisms depend on each other, for example through the production/consumption of metabolites by the partner organisms (Bryant *et al.*, 1967; Schink and Stams, 2013; Dolfing, 2014). Often, this phenomenon is called "syntrophy" or "cross-feeding". These obligate mutualistic interactions have been described in different microbial consortia (Bryant *et al.*, 1967; Schink, 1997; Drake *et al.*, 2009) and are primarily found in ecosystems that lack oxygen as the terminal electron acceptor (Morris *et al.*, 2013). One example is the cross-feeding on hydrogen (H_2) that is accumulation during the degradation of amino acids. In high concentrations H_2 can hamper the growth of the degrading bacterium. However, the subsequent metabolization of H_2 by other bacteria like *Methanospirillum hungatei* and *Acetobacterium woodii* significantly improves the growth of the amino acid degrading bacterium (Zindel *et al.*, 1988). Similarly, the process of nitrification from ammonia (NH_3) to nitrate (NO_3^-) by two groups of organisms is an example for cooperation of microorganisms: In the first step NH_3 is oxidized to nitrite (NO_2^-) which is subsequently utilized by NO_2^--oxidizing bacteria and converted to NO_3. Nitrification plays an important role in wastewater treatment facilities but also in surface- water and groundwater ecosystems (Ward, 1996; Sorokin *et al.*, 2012; Opitz *et al.*, 2014; Chen *et al.*, 2016; Wegner *et al.*, 2019).

The loss of "metabolic autonomy at the expense of entering a functional dependence" on other microorganisms still lacks evolutionary explanation (Pande and Kost, 2017). Indeed, competition instead of cooperation is dominating the interactions between cultured microorganisms (Foster and Bell, 2012). On the other hand, it has been shown that especially between yet uncultured microorganisms, the majority of bacteria lack key genes to synthesize central metabolites, suggesting cooperative interactions should be more common than

previously assumed (D'Souza *et al.*, 2014). Yet, the relevance of cooperative interactions in the terrestrial subsurface and the mechanisms behind them are only poorly understood.

1.1.2 Trophic Interactions

Beside interactions via metabolites, microorganisms are also part of complex food webs and thus involved in trophic interactions with other microorganisms and higher organisms. In marine and lake ecosystems, the role of bacteria in the food web has been widely acknowledged in the concept of the "microbial loop" (Azam *et al.*, 1983; Fuhrman, 1999). Photosynthesis-driven primary production is providing fresh organic carbon to the ecosystem and heterotrophic bacteria subsequently use detritus as a carbon source. The microbial biomass is then consumed by bacterivorous protozoan and metazoan organisms. Like this, carbon is transferred through the food chain, and ultimately again recycled by bacteria.

In ecosystems that lack photosynthetic primary production, like groundwater, microorganisms depend on the scarce input of organic matter (OM) from the surface (Baker *et al.*, 2000; Foulquier *et al.*, 2009), chemolithoautotrophic primary production (Hutchins *et al.*, 2016) or organic carbon stored in the rock matrix (Krumholz, 2000; Nowak *et al.*, 2017). CO_2-fixation by prokaryotes is of great importance for subsurface carbon cycling and sets the base of the microbial food web in groundwater ecosystems (Akob and Küsel, 2011). Chemolithoautotrophic microorganisms, that obtain energy by oxidizing inorganic compounds like hydrogen, ammonia or ferrous iron, are primary producers that generate biomass independent from OM inputs from the surface. Thereby, chemolithoautotrophic bacteria and archaea play a crucial role at the bottom of the microbial food webs in a variety of aquatic ecosystems like the deep-sea, lakes or rivers (Lonsdale, 1977; Kohzu *et al.*, 2004; Casamayor *et al.*, 2008). Recently, the relevance of chemolithoautotrophic primary production has also been demonstrated in groundwater of a karst aquifer, where it accounts for up to 69 % of the OM metabolized by the groundwater fauna (Hutchins *et al.*, 2016). Both bacterivorous protozoa and predatory bacteria feed on other bacteria and thereby mediate carbon fluxes from primary producers and consumers to higher trophic levels (Stolp, 1973; Risse-Buhl *et al.*, 2013; Hutchins *et al.*, 2016).

Besides the in-situ primary production of fresh biomass, microorganisms can also recycle dead microbial biomass (necromass) that is either introduced to the groundwater from the surface, or a result of cell death in the subsurface. Bacterial necromass has been shown to be ubiquitously distributed in soils and can, subsequently, be transported to the groundwater (Miltner *et al.*, 2012; Gleixner, 2013; Ma *et al.*, 2018). In addition, the nutrient limited conditions in the

groundwater might lead to the death of bacterial cells that enter the aquifer after being transported through soils and seepage water. As a result, members of the microbial community that persist or even flourish in groundwater are well adapted to the oligotrophic conditions in groundwater and may be exploiting necromass as a source of organic carbon compounds as well as cell components like amino acids, nucleotides or lipids (Orsi *et al.*, 2018; Starr *et al.*, 2018). The use of necromass as a carbon source has been proposed and demonstrated for bacteria in different aquatic ecosystems, including microorganisms that lack cultivated representatives (Müller *et al.*, 2018; Orsi *et al.*, 2018; Starr *et al.*, 2018) and necromass recycling has been demonstrated within an enrichment culture of a hydrocarbon degrading consortium where fermentative bacteria utilize dead bacterial biomass and thereby provide electron donors to the hydrocarbon-degrading bacteria. Based on these findings, the existence of a "subsurface microbial loop" has been proposed (Dong *et al.*, 2018).

1.1.3 Interactions Between yet Uncultivated Microorganisms

While the mechanisms of microbial interactions for many groups of bacteria are well understood and studied, aquatic ecosystems also harbour a wide range of microbial diversity whose interactions with other microorganisms are believed to be obligatory, yet poorly understood. Groundwater systems have been found to contain a particularly high fraction of microorganisms belonging to phyla that lack cultivated representatives including members of the Candidate Phyla Radiation (CPR, also referred to as *Cand.* Patescibacteria (Parks *et al.*, 2018); Bruno *et al.*, 2017; Kumar *et al.*, 2017; Schwab *et al.*, 2017). However, the role of CPR in the environment and their lifestyle can only be assumed based on available genomes.

The small genomes of CPR bacteria are generally characterized by the lack of genes for amino acid, nucleotide and lipid synthesis (Brown et al., 2015; Luef et al., 2015; Castelle and Banfield, 2018; Probst et al., 2018a). It is generally assumed that CPR are symbiotic members of the microbial community (Luef *et al.*, 2015). Cultivation-based studies support the hypothesis of a partner-dependent lifestyle of the CPR: The Parcubacterium *Cand.* Sonnebornia yantaiensis has been described as an intracellular symbiont of the protist *Paramecium bursaria* (Gong *et al.*, 2014). Together with the alga *Chlorella*, *Cand*. Sonnebornia yantaiensis and *Paramecium* form a three-partner consortium in a freshwater pond (Gong *et al.*, 2014). Furthermore, a member of the CPR phylum *Saccharibacteria* (TM7x) was isolated as an obligate epibiotic symbiont of *Actinomyces odontolyticus* (He *et al.*, 2015).

Interactions between different trophic levels or symbiotic partner organisms potentially have great implications on the structure of the groundwater microbiome and the entire groundwater fauna. However, the types of metabolic- and trophic interactions between groundwater microorganisms and the consequences that these interactions have for the transfer of carbon within the microbial community in subsurface ecosystems is to date only poorly understood.

1.2 Assessing the Metabolic Potential of Microorganisms

Microbial communities are highly complex and investigating the metabolic potential of the bulk community or individual members is not trivial, as the metabolism of an organism is often partially influenced by interactions with other organisms. A combination of cultivation-dependent and -independent techniques is needed to get the most complete picture of the metabolic potential of microorganisms in natural communities as possible.

1.2.1 Potential and Limitations of Cultivation-based Techniques

In order to fully assess the metabolic potential and physiology of a microorganism, cultivation-based studies using microbial isolates are essential. Only the availability of a microorganism, or at least a consortium of microorganisms in the form of enrichment cultures allows to fully describe the actual capabilities of individual strains. The screening of bacterial isolates for their potential to produce antibiotics is only one example of the importance of cultivation-based work (Tyc *et al.*, 2014). While studies have shown that the production of antimicrobial compounds can be increased when co-cultivating different bacteria, these methods still lack high-throughput cultivation and automated screening methods to check for the production of antimicrobials (Tyc *et al.*, 2014, 2017). Besides being of interest in the search for novel natural products for drug design, elucidating the potential to produce growth affecting secondary metabolites is also of relevance for understanding microbial interactions in the environment. Thus, novel cultivation and screening methods for natural products are promising developments in both environmental and clinical microbiology (Mahler *et al.*, 2018; Cross *et al.*, 2019; Wiegand *et al.*, 2019).

With an increasing amount of sequencing data from environmental samples, this gap between cultivated microbes and the microbial diversity present in the environment is becoming even more striking. Within the domains of Archaea and Bacteria, entire phyla that lack cultivated representatives have been discovered (Brown *et al.*, 2015; Spang *et al.*, 2015; Anantharaman *et*

al., 2016), demonstrating the need to combining cultivation-dependent and -independent methods for a better understanding of microbial communities, their functioning and evolutionary histories (Zaremba-Niedzwiedzka *et al.*, 2017; Imachi *et al.*, 2019).

1.2.2 Cultivation-independent Techniques

Amplicon-based Techniques. Amplicon-based techniques like quantitative PCR (qPCR) of functional marker genes or high throughput sequencing, can give first insights into the metabolic potential of complex communities. By quantifying marker genes of certain processes, like the genes encoding the key enzyme for CO_2-fixation via the Calvin-Benson-Bassham cycle, RubisCO, assumptions on potential processes taking place can be made (Alfreider *et al.*, 2003; Tabita *et al.*, 2007). If the quantification of these marker genes is made on transcript level, information about the actual expression of these genes by the microbial community can be gained.

The sequencing of phylogenetic- (e.g. 16S / 18S rRNA genes) and functional marker genes allows to generate an overview of the phylogenetic or functional composition of environmental communities (**Figure 2A**). The large datasets that can be generated with this method have now almost entirely replaced methods like molecular cloning and allow an in-depth analysis of complex microbial communities. Estimates of relative abundances of certain groups of microbes in a sample for example allow the analysis of community dynamics over time or the detection of rare taxa (Yan *et al.*, 2019). However, like all PCR-based methods, high-throughput sequencing of amplicons is not immune to primer biases. Around 10 % of the microbial diversity in environmental samples are missed by PCR-based sequencing techniques (Eloe-Fadrosh *et al.*, 2016). Especially microbes of yet uncultivated phyla like the CPR and novel groups of Archaea are frequently overlooked by amplicon sequencing approaches as insertions in their 16S rRNA gene (Brown *et al.*, 2015) or mismatches with commonly used primer sets impede their detection (Eloe-Fadrosh *et al.*, 2016).

Meta- and Single-Cell Genomics. Unlike PCR-based high-throughput sequencing methods, shotgun sequencing of environmental DNA is not affected by the use of PCR primers that are biased towards or against certain taxa (Eloe-Fadrosh *et al.*, 2016). Thus, shotgun metagenomics enables a more complete picture of the diversity within environmental samples but also the overall metabolic potential of a community (**Figure 2A**). The development of new algorithms and bioinformatic tools for assembling metagenomic sequence reads to larger contigs as well

as methods for binning these contigs into groups of sequences that belong to the same organism furthermore results in the recovery of more and more metagenome assembled genomes (MAGs). These MAGs allow us to draw conclusions about the metabolic potential of single organisms within an environment, broaden our knowledge about general microbial diversity on Earth and even challenge evolutionary theories, such as the evolution of eukaryotic life (Anantharaman *et al.*, 2016; Hug *et al.*, 2016; Eme *et al.*, 2017; Parks *et al.*, 2017; Zaremba-Niedzwiedzka *et al.*, 2017).

Despite their value for microbial ecology, MAGs are population genomes, meaning that within the process of sequence assembly and binning, sequences that origin from different cells can be concatenated and combined to a genome due to their sequence similarity. In nature, however, populations of the same species can exhibit a high degree of genomic heterogeneity (Rinke *et al.*, 2014). Cell-sorting based single-cell genomics, that allow the sequencing of genomes from individual cells (single amplified genomes (SAGs)) circumvent the problem of cross-assembly of multiple strains and thus are a powerful tool to explore the full metabolic potential of uncultivated microorganisms in an environment (Stepanauskas, 2012; Rinke *et al.*, 2014). In addition, genome-centric methods have been used to improve cultivation methods and have led to the successful cultivation of members of the CPR (Cross *et al.*, 2019).

Stable Isotope Probing. Unlike genomics-based techniques, that can only point towards potential functions that can be carried out by an organism or a community, methods based on stable isotope probing (SIP) allow to link taxonomic identification to metabolic activity of that organism through the assimilation of an isotopically labelled substrate in complex communities (Radajewski *et al.*, 2000). In brief, a stable isotope containing substrate is added to an incubation (**Figure 2B**). Organisms that utilize the labelled substrate take up the isotopic label and incorporate it in their biomass, including their DNA, RNA and proteins. Labelled and unlabelled nucleic acids are separated by density gradient centrifugation and individually sequenced (Radajewski *et al.*, 2000). The proteins can be identified and analysed by mass spectroscopy and subsequently their taxonomic affiliation is assigned using reference databases (Taubert *et al.*, 2012).

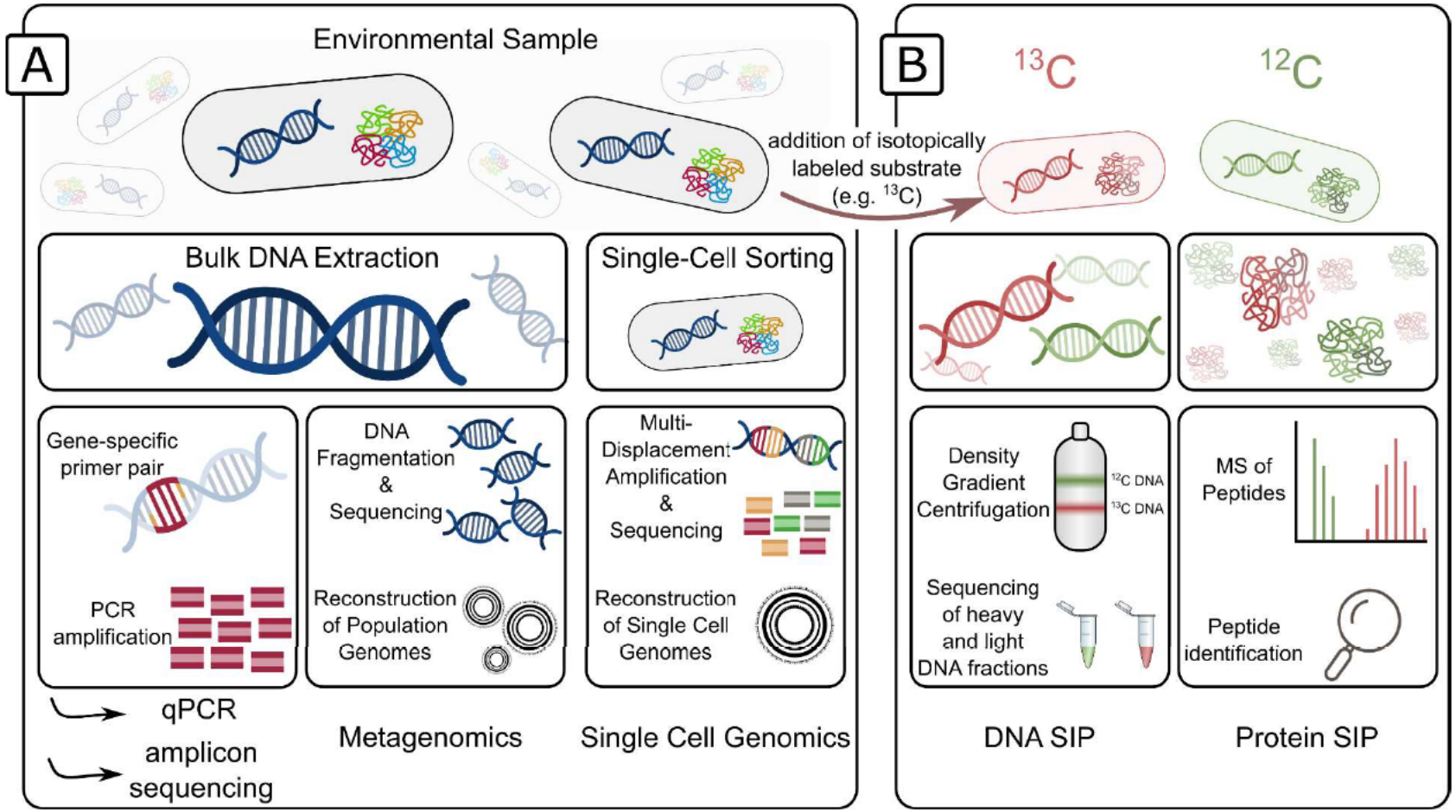

Figure 2 | Schematic overview of the introduced cultivation-independent methodology.

(A) Quantification and characterization of the microbial community in an environmental sample by PCR-based (qPCR & amplicon sequencing) and PCR-free methods (metagenomics & single cell genomics). (B) DNA- and Protein-SIP allow conclusions about the utilization of a specific substrate by following the incorporation of labelled substrates into the DNA or proteins of the respective organisms.

1.3 Groundwater as an Ecosystem

Groundwater is defined as subsurface water that "connectedly fills the Earth crust's voids and whose fluctuation is exclusively or almost exclusively governed by gravity and the caused friction forces" (DIN 4049-1, 1992). More than 30% of the fresh water on Earth is retained in the terrestrial subsurface (Loquay *et al.*, 2009; Hölting and Coldewey, 2013). Not only does groundwater play an important role in the global water cycle, but it also provides crucial ecosystem services like the supply of drinking water. Karst aquifers for example provide almost 25 % of the worlds drinking water (Ford and Williams, 2007). The absence of light, hampering primary production via photosynthesis, as well as a limited input of organic carbon, distinguish groundwater ecosystems from other subsurface ecosystems like soils, or surface aquatic ecosystems (Griebler and Lüders, 2009). These unique features of groundwater as a habitat for life turn aquifers into a challenging environment for microorganisms (Danielopol *et al.*, 2000; Griebler and Lüders, 2009). Additionally, agricultural intensification and the accompanied introduction of nitrogen and phosphorus derived from fertilizers poses a major threat to water quality. The natural conditions within the groundwater but also a degradation of the water quality make it a challenging environment for microbial life.

1.3.1 Microbial Life in Groundwater

With 93 Gt of carbon microorganisms (Bacteria, Archaea, Protozoa and Fungi) are the second largest biomass component on Earth (Bar-On *et al.*, 2018). Bacteria account for the biggest portion of microbial biomass, followed by fungi, Archaea and protists (Bar-On *et al.*, 2018). The majority of microbial biomass is stored in the terrestrial subsurface, including the deep continental subsurface, soils and groundwater (Bar-On *et al.*, 2018; Flemming and Wuertz, 2019).

Prokaryotes. Abundances of prokaryotes in groundwater vary drastically between different aquifer systems. In karst aquifers, bacteria reach abundances of up to 10^8 cells L^{-1}, while archaeal abundances range between 10^3 and 10^5 cells L^{-1} (reviewed in Akob and Küsel, 2011; Opitz *et al.*, 2014; Lazar *et al.*, 2017; McMahon and Parnell, 2014). Besides the comparably low cell numbers, the oligotrophic conditions in many groundwater systems often favour groups of microorganisms with particular small cell sizes (Luef *et al.*, 2015). The increased surface-to-volume ratio of smaller cells for example leads to a more efficient uptake of the limited nutrients in groundwater (Sowell *et al.*, 2009). At the same time, a lack of nutrients can also lead to a

decreasing cell size in starving bacteria (Hood and MacDonell, 1987; Vybiral *et al.*, 1999; Young, 2006). Furthermore, small cells sizes are often correlated with smaller sized genomes. These reduced genomes lower the metabolic costs of reproduction for the bacterium and are thus advantageous in low-nutrient systems (Giovannoni *et al.*, 2014). Ultra-small bacteria with cell sizes smaller than 0.2 µm and streamlined genomes that lack critical metabolic functions are found to be highly abundant in groundwater ecosystems (Wrighton *et al.*, 2012; Brown *et al.*, 2015; Luef *et al.*, 2015). However, as these bacteria are challenging with respect to cultivation, little is known about their role in the environment, interactions with other microorganisms or their full metabolic potential in subsurface habitats.

With the advances in microbial genomics and sequencing techniques, the tree of life recently underwent a remarkable expansion within both prokaryotic domains, Bacteria and Archaea (Brown *et al.*, 2015; Hug *et al.*, 2016; Parks *et al.*, 2017; Zaremba-Niedzwiedzka *et al.*, 2017; Castelle and Banfield, 2018). Genome-resolved metagenomics and single-cell sequencing have contributed significantly to our knowledge about the metabolic potential of microorganisms and the extent of their diversity in various difficult to access environments, including groundwater (Brown *et al.*, 2015; Luef *et al.*, 2015; León-Zayas *et al.*, 2017). With their "New View of the Tree of Life" Hug and colleagues (2016) illustrated the amount of bacterial and archaeal phyla that to date still lack cultivated representatives (Candidate Phyla), by highlighting the diversity within an entire radiation in the tree of life that almost entirely lacks cultivated members: the Candidate Phyla Radiation (CPR) (Hug *et al.*, 2016; Castelle and Banfield, 2018).

Eukaryotes. Despite the limitations in space and nutrients for microeukaryotes, protozoa have been shown to inhabit shallow as well as deep groundwater systems (Griebler and Lüders, 2009; Akob and Küsel, 2011; Griebler and Avramov, 2015). Eukaryotic 18S rRNA gene abundances in groundwater vary between 1.0×10^5 and 4.0×10^6 gene copies L^{-1} (Loquay *et al.*, 2009; Akob and Küsel, 2011; Risse-Buhl *et al.*, 2013). However, studies investigating their diversity are rare (Novarino *et al.*, 1997) and seldomly based on culture-independent observations. Reports on protozoan diversity in groundwater of a karstic limestone system confirm a diverse micro-eukaryotic presence, including ciliates, flagellates, amoebae, heliozoans and sporozoans (Novarino *et al.*, 1997; Risse-Buhl *et al.*, 2013). These findings indicate the presence of mainly bacterivorous protozoa, but also flagellates and heliozoans, known to also feed on other protozoa. Higher organisms, metazoans, have equally been reported to be inhabiting

groundwater ecosystems. However, their presence has been reported to be tied to oxic conditions in the groundwater, as well as the presence of other microorganisms to feed on (Griebler and Mösslacher, 2003; Hahn, 2006). Thus, food chains in the terrestrial subsurface are often assumed to be short and of low species diversity within the different trophic levels (Gibert and Deharveng, 2002; Hüppop, 2012; Hutchins *et al.*, 2016).

1.3.2 Field Site

The Hainich Critical Zone Exploratory (CZE) has been established within the "ProExzellence" initiative AquaDiva@Jena and the consecutive collaborative research centre (CRC)1076 AquaDiva. The central aim of the CRC AquaDiva is to understand how surface conditions, such as management types, weather events or geology, influence and shape the functional biodiversity of the subsurface (Küsel *et al.*, 2016). The field site is located in central Germany in close proximity to the Hainich national park (Küsel *et al.*, 2016). Two superimposed karstic limestone aquifer assemblages, following a downhill slope, can be investigated along a ~ 6 km transect (Küsel *et al.*, 2016; Kohlhepp *et al.*, 2017; **Figure 3A**).

Both aquifers are accessed at five sites along the transect through fifteen groundwater wells (**Figure 3A**). The unique design of the groundwater wells, with wide slots in the well screens and coarse filter material surrounding the wells, minimizes the attachment of particles and organisms to the filter material (Küsel *et al.*, 2016) and at the same time allows larger organisms to pass the filter and enter the groundwater wells (**Figure 3C**). Lysimeters enable a regular sampling of seepage water that percolates the soils and ultimately contributes to groundwater recharge (**Figure 3A**). By that, the transport of microorganisms and matter from the surface to the subsurface can be monitored, contributing to an improved understanding of the formation of the groundwater microbiome. Since the establishment of the Hainich CZE, the hydrochemical parameters (Kohlhepp et al., 2017) and microbial inventory of the groundwater have been continuously monitored (Risse-Buhl *et al.*, 2013; Herrmann *et al.*, 2015, 2017; Kumar *et al.*, 2017; Yan *et al.*, 2019). The two aquifer assemblages, as well as the different sites, significantly differ in the hydrochemistry of the groundwater (**Figure 3B**)

The analysis of metagenomic and metatranscriptomic data from both aquifers revealed a broad functional diversity in the groundwater microbial community that is mainly driven by nitrogen and sulfur cycling (Wegner *et al.*, 2019). Generally dominated by *Cand.* Patescibacteria, *Proteobacteria* and *Nitrospirae* (Kumar *et al.*, 2017; Schwab *et al.*, 2017; Yan *et al.*, 2019) the

core microbiome consists of 26 bacterial operational taxonomic units (OTUs) that persist across space and time (Yan *et al.*, 2019). Besides redox potential and pH in the upper aquifer assemblage and total inorganic carbon and nitrite in the lower aquifer assemblage, ammonium concentrations are driving the community composition in the groundwater (Yan *et al.*, 2019).

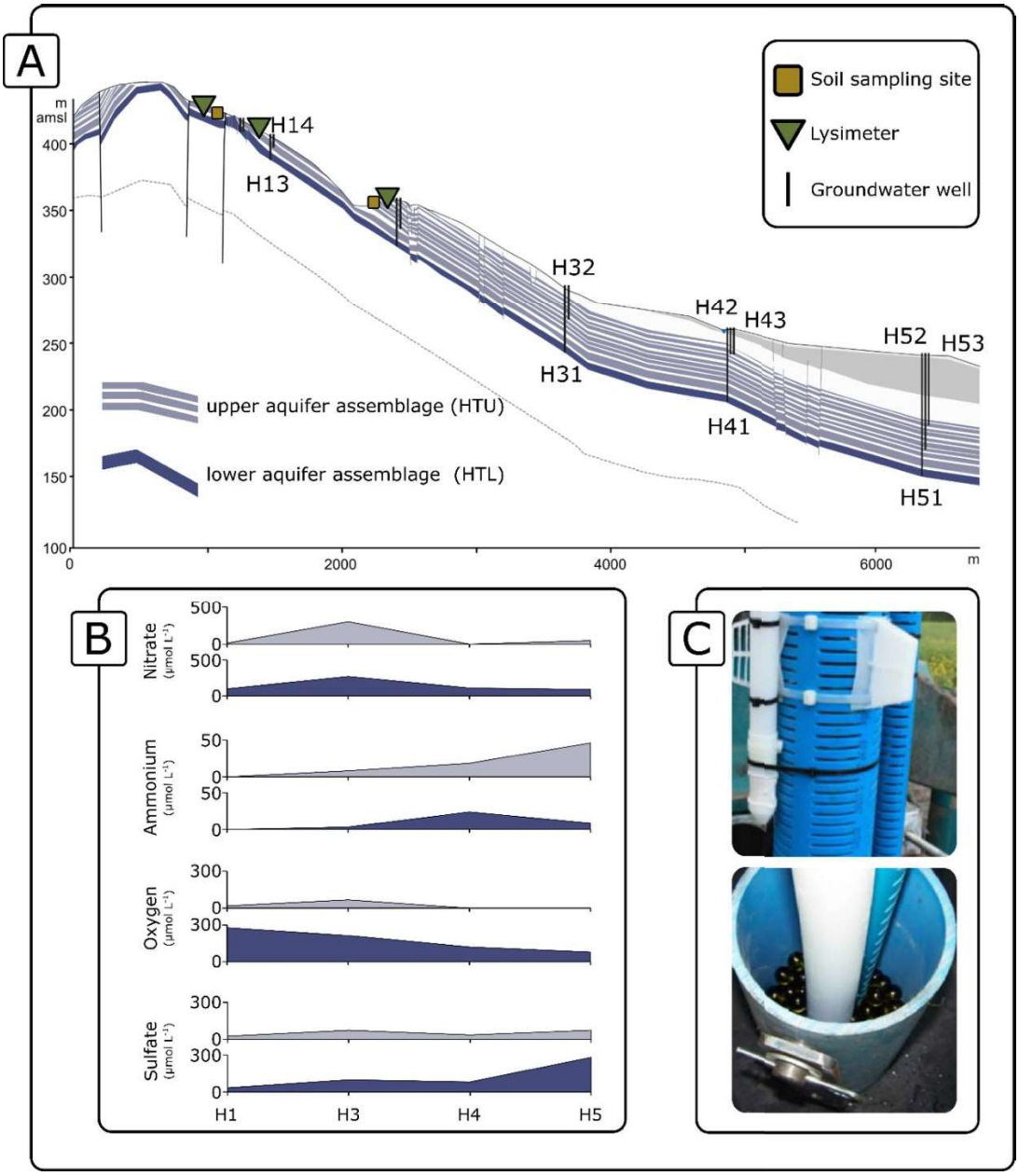

Figure 3 | Overview of the Hainich Critical Zone Exploratory groundwater well transect.
(A) Schematic illustration of the transect including the locations of groundwater wells, lysimeters and soil sampling locations (modified from Kohlhepp *et al.*, 2017) facilitate to follow the groundwater flow path (**Chapter 3, 4 & 6**). (B) The groundwater hydrochemistry in both the upper (HTU) and lower (HTL) aquifer assemblage across the different sampling sites (H1, H3, H4, H5). (C) Photographs depicting the unique well design with wide slots in the well tubing as well as coarse glass beads used as inert backfilling material (modified from Küsel *et al.*, 2016) enable the sampling of larger, eukaryotic community members (**Chapter 5 & 6**).

1.4 Hypotheses and Objectives

Bacteria inhabiting oligotrophic groundwater are known to often lack critical metabolic functions. In order to lower their individual metabolic expenses cooperation might be of more relevance than antagonistic interactions. One example of a group of groundwater microorganisms whose restricted metabolic capacity presumably leads to a dependency on other microorganisms are members of the CPR. Beside the large fraction of CPR, autotrophic microorganisms have been found to play an important role in the groundwater microbiome of the Hainich CZE and could be a partner for CPR. Therefore, the first two hypotheses of this thesis were:

I. Positive interactions between microorganisms prevail in groundwater ecosystems

II. Fermentative members of the CPR are linked to autotrophic partners in the groundwater microbiome

In order to address these hypotheses, the objectives were:

i. Development of a standardized high-throughput method to screen a broad selection of bacterial isolates for growth-inhibiting and -promoting effects in liquid culture.
ii. Detailed analysis of the genome of one CPR bacterium to identify potential host association factors.
iii. Prediction of interactions between yet uncultivated members of the CPR with other community members using co-occurrence networks.

As the input of photosynthesis-derived organic matter into the groundwater is limited, the groundwater microbiome is adapted to oligotrophic conditions. Carbon can be provided by autotrophic bacteria as well as through the recycling of necromass. Thus, the third hypothesis of this thesis was:

III. Groundwater microorganisms respond to the availability of different carbon sources

To test this third hypothesis, the objectives were:

iv. Analysis of the response in cell size of groundwater bacteria to different concentrations of organic carbon.
v. Tracking of the uptake of necromass by the groundwater microbiome using SIP.
vi. Elucidation of the genetic potential for chemolithoautotrophic primary production in groundwater.

1.5 Structure of the Thesis

Within the following five chapters, the hypothesis and aims of this dissertation will be addressed (**Figure 4**).

In Chapter 2 **"Growth promotion and inhibition induced by interactions of groundwater bacteria"** (Geesink *et al.*, 2018; published in FEMS Microbiology Ecology) the effects that bacterial isolates from groundwater grown in mono- and co-cultures have on a the growth of a third bacterium were investigated. The newly developed method allowed the screening for both, growth promotive and inhibitive effects in liquid media. This work has been carried out in collaboration with Paolina Garbeva, Olaf Tyc and Charlotte van de Velde (NIOO-KNAW, Wageningen, The Netherlands).

Chapter 3 **"Predominance of *Cand.* Patescibacteria in groundwater is caused by their preferential mobilization from soils and flourishing under oligotrophic conditions"** (Herrmann *et al.*, 2019; published in Frontiers in Microbiology) elucidated the origin of ultra-small bacteria belonging to the CPR in groundwater and their co-occurrence patterns with other microorganisms. Furthermore, the effects of starvation on cell size of heterotrophic bacteria isolated from groundwater were demonstrated.

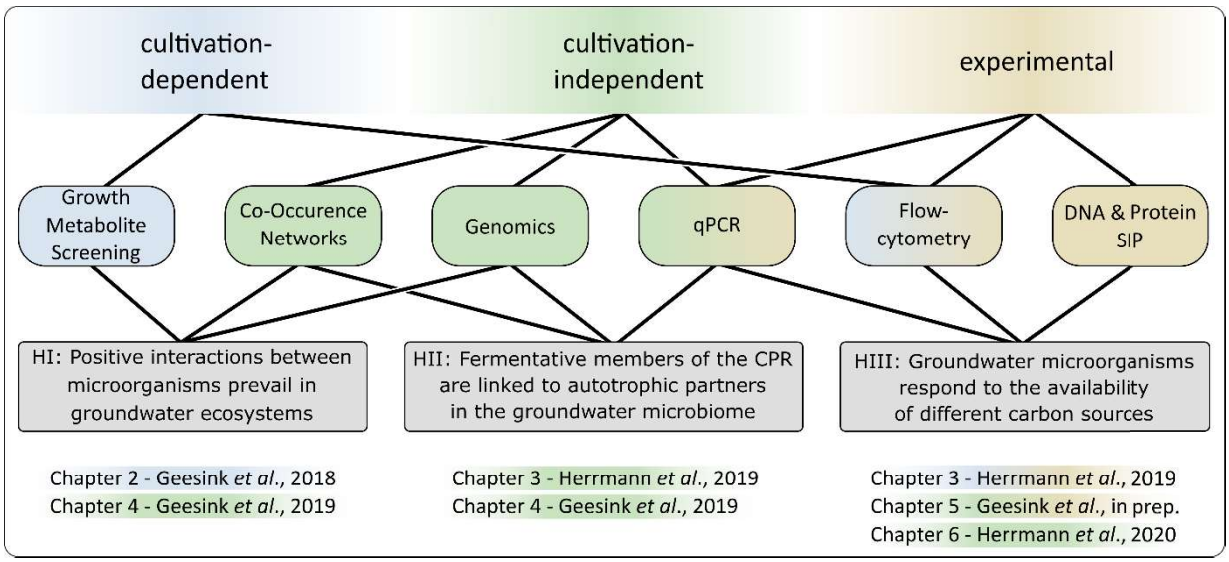

Figure 4 | Workflow to investigate the three hypotheses (HI – HIII) of this dissertation. Cultivation-dependent (blue), cultivation-independent (green) and experimental approaches (orange) were used in combination with different methods in microbial ecological. All three hypotheses are discussed in the following chapters of this thesis.

Within Chapter 4 **"Genome-inferred spatio-temporal resolution of an uncultivated Roizmanbacterium reveals its ecological preferences in oligotrophic groundwater"** (Geesink *et al.*, 2019; published in Environmental Microbiology) the genome of a member of a yet uncultivated Roizmanbacterium was analysed. To gain further insight into its ecological preferences its abundance was tracked across a transect of multiple groundwater wells over the course of one year. This work was conducted in collaboration with Alexander Probst (University Duisburg-Essen) as well as Hang Dam and Anne-Kristin Kaster (KIT Karlsruhe).

In Chapter 5 **"Bacterial necromass is rapidly metabolized by heterotrophic members of the microbial community in groundwater"** (Geesink *et al.,* in preparation) the uptake of ^{13}C labelled necromass by the bacterial and eukaryotic community was followed using DNA- and Protein-SIP techniques. A rapid metabolization of amino acids by heterotrophs stimulates autotrophic community members as well as eukaryotic members of the groundwater community. This work was conducted in collaboration with Nico Jehmlich and Martin von Bergen (UFZ Leipzig).

In Chapter 6 **"Complex food webs coincide with high genetic potential for chemolithoautotrophy in fractured bedrock groundwater"** (Herrmann *et al.,* 2020; published in Water Research) the genetic potential for bacterial chemolithoautotrophy was estimated and different levels of putative trophic interactions by pro- and eukaryotes in groundwater were disentangled. The results presented in this chapter disclose complex food webs within groundwater that coincide with an elevated genetic potential for chemolithoautotrophic primary production.

2. Growth promotion and inhibition induced by interactions of groundwater bacteria

Patricia Geesink, Olaf Tyc, Kirsten Küsel, Martin Taubert, Charlotte van de Velde, Swatantar Kumar and Paolina Garbeva

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Microorganisms can produce a plethora of secondary metabolites, some acting as signaling compounds and others as suppressing agents. As yet, the potential of groundwater microbes to produce antimicrobial compounds to increase their competitiveness against other bacteria has not been examined. In this study, we developed an AlamarBlue® based high-throughput screening method that allowed for a fast and highly standardized evaluation of both growth-inhibiting and -promoting metabolites. With this technique, 149 screened bacterial isolates were grown in monocultures and in 1402 co-cultures. Co-cultivation did not increase the frequency of growth inhibition against the two tested model organisms (*Staphylococcus aureus* 533R4 and *Escherichia coli* WA321) compared to monocultures. Mainly co-cultivation of Proteobacteria induced growth inhibition of both model organisms. Only slightly increased growth promotion of *S. aureus* 533R4 was observed. Growth-promoting effects on *E. coli* WA321 were observed by supernatants from co-cultures between Bacteroidetes and Firmicutes. With the standardized screening for both growth-inhibiting and -promoting effects, this method will enable further studies to elaborate and better understand complex inter-specific interactions and networks in aquatic communities as well as in other environments.

Supplementary data to this article can be found online at https://academic.oup.com/femsec/ article-lookup/doi/10.1093/femsec/fiy164#supplementary-data

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Research Article

RESEARCH ARTICLE

Growth promotion and inhibition induced by interactions of groundwater bacteria

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One sentence summary: A newly developed high-throughput screening method for growth inhibition and growth promotion induced by groundwater bacteria opens a new door to understanding the complex interactions in microbial communities.

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ABSTRACT

Microorganisms can produce a plethora of secondary metabolites, some acting as signaling compounds and others as suppressing agents. As yet, the potential of groundwater microbes to produce antimicrobial compounds to increase their competitiveness against other bacteria has not been examined. In this study, we developed an AlamarBlue® based high-throughput screening method that allowed for a fast and highly standardized evaluation of both growth-inhibiting and -promoting metabolites. With this technique, 149 screened bacterial isolates were grown in monocultures and in 1402 co-cultures. Co-cultivation did not increase the frequency of growth inhibition against the two tested model organisms (*Staphylococcus aureus* 533R4 and *Escherichia coli* WA321) compared to monocultures. Mainly co-cultivation of Proteobacteria induced growth inhibition of both model organisms. Only slightly increased growth promotion of *S. aureus* 533R4 was observed. Growth-promoting effects on *E. coli* WA321 were observed by supernatants from co-cultures between Bacteroidetes and Firmicutes. With the standardized screening for both growth-inhibiting and -promoting effects, this method will enable further studies to elaborate and better understand complex inter-specific interactions and networks in aquatic communities as well as in other environments.

Keywords: groundwater bacteria; high-throughput screening; microbial interactions; secondary metabolites

INTRODUCTION

In nature, bacteria exist as part of a community where they are constantly interacting with their own and other bacterial species. Bacterial growth and performance depend highly on these interactions (Hibbing *et al.* 2010; Braga, Dourado and Araújo 2016). Thus, bacterial interactions play an important role in the microbial community composition and functioning (Pande and Kost 2017). Bacteria frequently release primary and secondary metabolites into their environment and thereby interact with other microbes. Syntrophic relationships, where the primary metabolism of one organism is directly built on the primary metabolic products of another, are well-described phenomena occurring in the environment (Dolfing 2014; Pande and Kost 2017). Secondary metabolites, like antibiotics or growth factors, can have important ecological functions as they can both

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negatively or positively influence the growth of other bacteria in the same environment (Hibbing *et al.* 2010; Cornforth and Foster 2013; Pande and Kost 2017). In this way, they not only modify their niche but can also affect the performance and secondary metabolite production of other bacteria nearby (Seyedsayamdost *et al.* 2012; Pande and Kost 2017).

One of the most complex ecosystems, harboring a large diversity and density of bacteria, is the soil, with prokaryotic abundances ranging from 10^7 to 10^{10} cells/g soil (Uroz *et al.* 2010; Akob and Küsel 2011). In a heterogeneous and nutrient-poor soil environment, microorganisms may encounter and interact with numerous taxonomically different neighbors in different microsites in the soil. The production of antimicrobial compounds in the soil can be a useful strategy against competitors for nutrients (Demoling, Figueroa and Bååth 2007; Rousk and Bååth 2007; Rousk, Demoling and Bååth 2009). Recent studies focusing on soil bacteria revealed that the production of secondary metabolites is often a direct result of interactions with other microorganisms in their environment (Garbeva *et al.* 2011; Traxler *et al.* 2013; Tyc *et al.*, 2014, 2017). For example, bacteria of the genus *Streptomyces*, which produce over 70% of all naturally occurring antibiotics, have been found to exhibit more inhibitory effects in pairwise interactions (Kinkel *et al.* 2014). Genomic studies revealed that in many microorganisms, gene clusters for secondary metabolite production are only expressed during certain biotic interactions or after receiving signals from neighboring microorganisms (Scherlach and Hertweck 2009; Cornforth and Foster 2013). Nonetheless, the production of secondary metabolites is assumed to be energetically expensive and therefore might be reduced or abandoned under nutrient-limited or -competitive conditions.

In freshwater ecosystems, research on the production of antimicrobial compounds is rare and has been restricted to studies with freshwater sponges (Keller-Costa *et al.* 2014) or surface water ecosystems. For example, almost 70% of bacteria isolated from the Amazon river were producing antimicrobial compounds against at least one of the seven tested indicator strains (Motta, Cladera-Olivera and Brandelli 2004). Groundwater, as a major reservoir for freshwater on our planet, is still insufficiently investigated in the context of microbial interactions via secondary metabolites. These subsurface environments are characterized by a generally low-nutrient content since no light driven primary production is present (Akob and Küsel 2011). With ~10^5–10^7 cells/L (Opitz *et al.* 2014), the abundance of bacteria in groundwater is at least three orders of magnitude lower than in surface water. Due to the lower cell numbers and the accompanying lower chances of direct cell contact, one could assume that interactions between bacteria in an aquifer are less likely to be of relevance. However, under nutrient-limited conditions in the subsurface, bacteria might significantly benefit from trading metabolites and may cooperate with respect to cost extensive production of exo-enzymes or secondary metabolites. In fact, groundwater communities are often dominated by bacteria with small, streamlined genomes of around 1 Mbp or less (Hug *et al.* 2016; Solden, Lloyd and Wrighton 2016) with reduced metabolic capabilities. Hence, the majority of these bacteria are proposed to live in symbiotic or parasitic association with other bacteria in the aquifer (Luef *et al.* 2015; Hug *et al.* 2016; Castelle *et al.* 2017) and are therefore not yet culturable. The dominance of these bacteria, with life strategies depending on inter- and intra-specific interactions, is pointing towards the decisive role of microbial interactions in groundwater ecosystems.

The aim of this study was to develop a standardized high-throughput method to screen bacterial isolates in liquid cultures for both growth-inhibiting and -promoting effects. The newly developed method is particularly suited for aquatic microbes and is applied here to test for the production of growth influencing metabolites during cultivation of monocultures and pairwise co-cultivation of groundwater bacterial isolates. We hypothesized that pristine groundwater is the perfect environment for microorganisms to evolve metabolic interactions that affect other microorganisms both negatively and positively.

MATERIALS AND METHODS

Origin of groundwater isolates

In the frame of this study, we used bacterial isolates obtained from pristine groundwater of a limestone aquifer. The aquifer is located in the Hainich region in northwest Thuringia where a groundwater monitoring transect, following a downhill slope, was established within the CRC AquaDiva (Küsel *et al.* 2016). Several groundwater wells access two superimposed aquifer assemblages that are either characterized by alternating sequences of fractured limestones (aquifers) and marlstones (aquitards). The used isolates were obtained from the lower, oxic aquifer. This groundwater is characterized by a dissolved oxygen concentration of around 6 mg/L, a pH of 7.2–7.5, a dissolved organic carbon concentration of around 2 mg/L, 10 mg/L nitrate and 0.1 mg/L ammonium (Küsel *et al.* 2016).

Isolation of groundwater bacteria and phylogenetic analysis

In order to obtain a set of representative bacterial isolates, several cultivation approaches using different media had been performed. For this study, we selected a subset of bacteria that were all able to grow in the same media, designed to reflect the low nutrient conditions of the groundwater. Ultimately, 149 bacterial isolates were selected and cultivated on a modified medium (Reasoner and Geldreich 1985) (0.6 g/L $K_2HPO_4 \cdot 3H_2O$, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.06 g/L Sodium pyruvate, 0.1 g/L peptone, 0.1 g/L Casein hydrolysate, 0.1 g/L Yeast extract, 0.1 g/L Dextrose, 15 g/L Agar-Agar, ultrapure), hereafter called S2P medium.

The phylogenetic affiliation of all isolates was determined by PCR amplification of the bacterial 16S rRNA gene fragment using the primer pairs Bac8F/907R (Lane 1991; Turner *et al.* 1999) amplifying ~899 bp from the 16S rRNA gene. Amplifications were performed on a thermocycler (Peqlab, primus96) with the following settings: initial denaturation temperature of 95°C for 5 min, followed by 30 cycles of 94°C for 45 seconds, 52°C for 60 seconds and 72°C for 60 seconds and a final elongation at 72°C for 10 minutes. Sequencing of the obtained PCR product was done using primer Bac8F (Macrogen, the Netherlands).

Subsequently all sequences were aligned in ARB, representative species of each bacterial family were picked from the SILVA database (release 123) (Pruesse *et al.* 2007) and the phylogenetic tree was calculated with the ARB neighbor-joining function (1000 bootstraps) and visualized using the interactive tree of life website (Letunic and Bork 2016). In order to estimate the abundance of every isolate within the groundwater bacterial community, we compared every isolate with a previously published 16S rRNA gene amplicon dataset (Schwab *et al.* 2017) on family level.

Target organisms

Escherichia coli WA321 (DSM 4509) and *Staphylococcus aureus* 533R4 Serovar 3 (DSM 20 231) were used as target organisms to screen

for the presence of growth-inhibiting or -promoting compounds, following Tyc *et al.* (2014). The target organisms were pre-cultured from −80°C glycerol stocks on LB agar plates (LB-Agar, Carl Roth, Germany) (Sambrook and Russell 2001) and incubated at 37°C. For the screening, single colonies of each target strain were picked from the plate and incubated at 37°C for 24 h in 25 mL LB medium (LB medium Lennox, Carl Roth GmbH + Co. KG, the Netherlands) prior to each screening assays.

Preparation of 96-well source plates

96-well microtiter plates (Greiner bio-one B.V., Alphen a/d Rijn, the Netherlands, Cat# 655 180) were used as source plates that contained all 149 bacterial isolates. Each well was filled with 300 µL liquid S2P medium and the bacterial isolates were inoculated in rows 3–10 manually by picking cells from a single colony of each bacterial isolate with an autoclaved wooden toothpick. The rows 1 and 2 as well as 11 and 12 were used for the negative and positive controls, containing 30 µL liquid S2P medium and 70 µL LB medium with AlamarBlue® solution (10% v/v) with or without target organism, respectively, within the actual screening run. In total, six different source plates (A–F) were produced and used for the screening. All source plates were generated in triplicates. The plates were incubated at room temperature for 6 days before the screening. In order to generate back-ups of each source plate, 50 µl of 50% (v/v) glycerol were added to prepare the plates for long-term storage at −80°C.

High-throughput interaction assay

A Genetix QPix 2 colony picking robot (Molecular Devices, UK Limited, Wokingham, UK) was used for the high-throughput interaction assay. The Genetix QPix 2 robot was mounted with a bacterial 96-pin picking head and programmed to replicate the provided source plates A–F in triplicates into 96-well microtiter plates supplemented with 300 µL per well liquid S2P medium. To maximize the number of co-cultures all plates were combined with each other in their original as well in an inverted orientation. The inoculated screening plates (monocultivation and co-cultivation plates) were incubated at 21°C for 6 days.

Screening for growth-inhibitory or -promoting effects of cell-free supernatants

After 6 days, the 96-well microtiter plates were centrifuged at 5000 rpm for 40 minutes at room temperature on a Sigma 3–14K laboratory centrifuge (SIGMA Laborzentrifugen GmbH, Germany) in order to retrieve cell-free supernatants from the bacterial isolates grown in monoculture or in co-culture.

For the screening, 96-well microtiter plates were prepared by dispensing 30 µL of the prepared cell-free supernatant into the screening plates. Then, a volume of 70 µL of the target organisms (either *E. coli* WA321 or *S. aureus* 533R4) grown in liquid LB Medium plus 10% (v/v) AlamarBlue® solution (Invitrogen™, Germany, cat# DAL1025) was added to each well, resulting in a total volume of 100 µL per well. The target organisms were added at a density of OD 0.004, corresponding to 6.4×10^6 CFU/mL (*E. coli* WA321) or 4.0×10^5 CFU/mL (*S. aureus* 533R4). The empty rows 1 and 2 were used as negative and positive controls. All Plates were incubated at 37°C for 4 hours according to the manufacturers manual. The AlamarBlue® solution was used to monitor the growth of the target organisms (Tyc *et al.* 2016), as a color change from blue to pink indicates metabolic activity. The metabolic activity was monitored using fluorescent measurements on a BioTek Synergy™ HT Multi-Mode Microplate Reader (Beun de Ronde Life Sciences, the Netherlands) applying excitation/emission wavelengths of 530 nm and 590 nm, respectively.

Data analysis and statistics

The relative growth of the target organisms in treatments with isolate or co-culture supernatant was compared to the growth of the control of the respective target organism without added supernatants. The supernatant of an isolate or co-culture was defined to have an inhibiting effect when the average of all triplicates showed 70% or less growth (lower fluorescence values) and to have a growth-promoting effect when there was on average 130% or more growth compared to the control (higher fluorescence values). To determine whether a particular taxonomic group, or the combination of two specific taxonomic groups, had a significantly higher tendency to provide effects on the growth of the target organisms, Fisher's exact test (Fisher 1922) was used for the calculation of *P*-values as

$$p = (a+b)! \times (a+c)! \times (b+d)! \times (c+d)! / (n! \times a! \times b! \times c! \times d!)$$

with a and b denoting the numbers of co-cultures tested including the taxonomic group(s) with (a) and without (b) the desired effect, c and d denoting the numbers of co-cultures excluding the taxonomic group(s) with (c) and without (d) the desired effect and n denoting the total number of co-cultures tested.

To display different levels of significance, *P*-value thresholds of <0.05 (*), <0.01 (**) and <0.001 (***) were used. Fisher's exact test was used in a likewise manner to determine whether the tendency of isolates showed an effect on the target organisms was significantly different from those of co-cultures.

RESULTS

Growth-inhibiting and -promoting effects of groundwater isolates

The selected 149 bacterial groundwater isolates represented 17 different bacterial families of the following four phyla: Actinobacteria (5), Bacteroidetes (35), Firmicutes (9) and Proteobacteria (11 Alphaproteobacteria, 10 Betaproteobacteria, 79 Gammaproteobacteria) (Genbank accession numbers MG980417-MG980565, Fig. 1; Fig. S1, Table S1, Supporting Information). These bacterial families were shown to cover 2.6% of the diversity present in the lower aquifer and represent 3.52% of the relative abundance of the groundwater bacterial community (Table S1, Supporting Information), as revealed by amplicon sequencing (Schwab *et al.* 2017).

We used the high-throughput screening approach to test for growth-inhibiting and -promoting effects of our isolates by transferring monoculture supernatants to the two target organisms *E. coli* WA321 and *S. aureus* 533R4. In most cases where changed growth of the target organisms was observed, isolates had an inhibitory effect. For example, when grown in monoculture 52 isolates showed inhibiting effects on the growth of the gram-negative *E. coli* WA321 target organism and 61 isolates on the growth of the gram-positive *S. aureus* 533R4 (Fig. 2A). Growth-promoting effects on *E. coli* WA321 were only observed with 18 isolates, and only 1 isolate (#134 belonging to the family Pseudomonadaceae) was promoting the growth of *S. aureus* 533R4 (Fig. 2A).

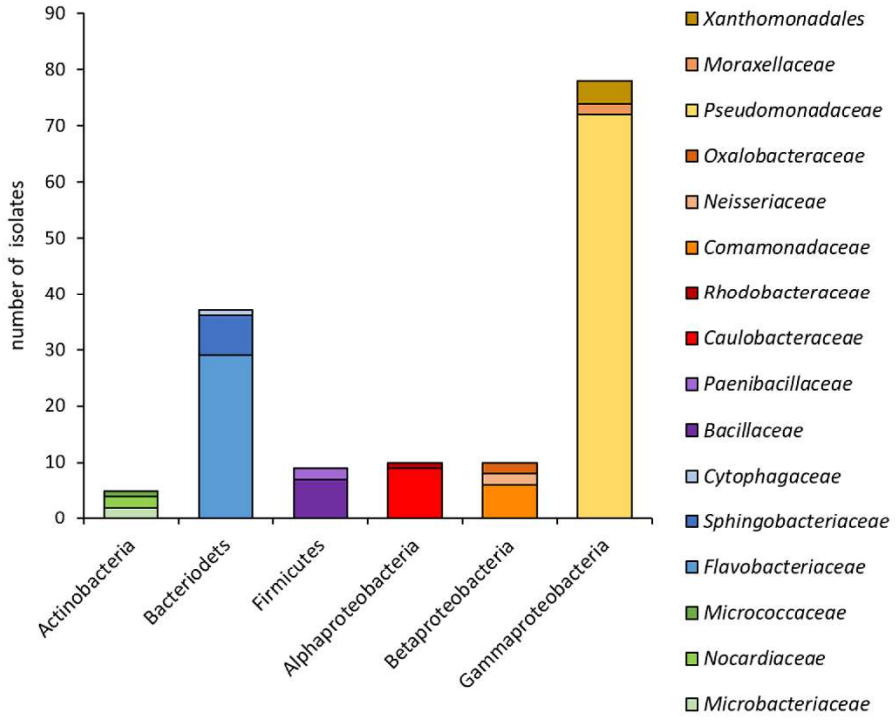

Figure 1. Overview of the 149 bacterial groundwater isolates belonging to sixteen different bacterial families within the phyla Actinobacteria (green), Bacteroidetes (blue), Firmicutes (purple) as well as Alpha- (red), Beta-(orange) and Gamma-(yellow) Proteobacteria.

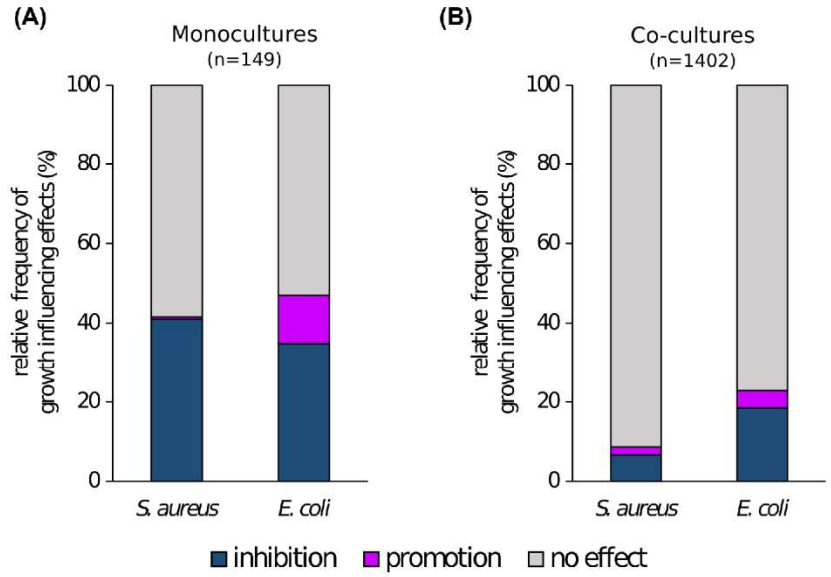

Figure 2. Relative fractions of growth promotion and inhibition in the tested monocultures (n = 149) and co-cultures (n = 1402). For both target organisms the relative occurrence of growth-influencing effects decreases in the tested co-cultures.

To test whether co-cultivation would show additional effects of the culture supernatant on the growth of the target organisms, we established 1402 random bacterial co-cultures with the 149 isolates. Interestingly, the fraction of co-cultures showing inhibitory effects on growth was significantly lower on both target organisms ($P < 0.001$, Fisher's exact test) than the fraction of monocultures. Less than 20% of co-cultures led to inhibitory effects on *E. coli* WA321 and for *S. aureus* 533R4 this number was below 10% (Fig. 2B). All phyla showed a significantly lower frequency of growth inhibition on *S. aureus* 533R4 ($P < 0.01$ to $P < 0.001$). For *E. coli* WA321 all phyla, except Proteobacteria, showed a significantly reduced frequency of inhibition ($P < 0.01$

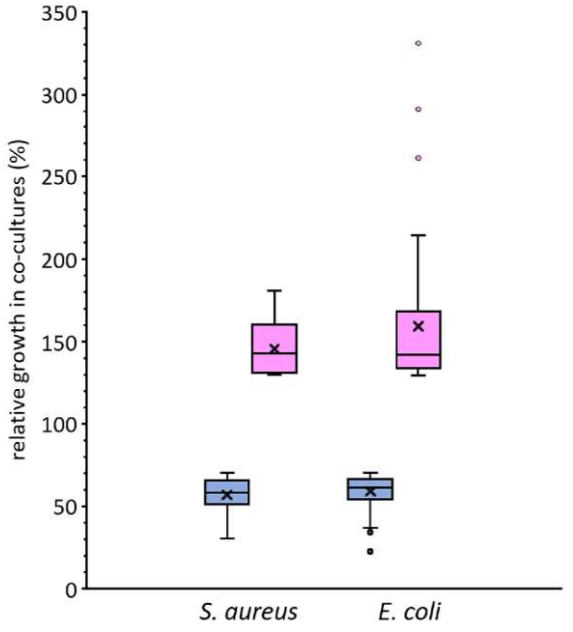

Figure 3. Relative growth of the two target organisms *S. aureus* 533R4 and *E. coli* WA321 in co-cultures that were evaluated to have growth-inhibiting (blue) and -promoting (pink) effects.

to $P < 0.001$) in co-cultures. For growth-promoting effects, the observed differences were not as clear. While for *E. coli* WA321 a significantly lower number ($P < 0.001$) of co-cultures stimulated growth, for *S. aureus* 533R4, the number of co-cultures leading to a growth promotion increased from 0.7% to 2%, although the difference was not significant (Fig. 2B). Hence, none of the investigated bacterial groups showed an increased frequency of growth-affecting activities in co-culture compared to monoculture.

The applied fluorometric assay further allowed us to quantify the growth-inhibiting and -promoting effects exhibited by the bacterial supernatants, and to compare them between monoculture and co-culture. The strength of both, growth inhibition and promotion, did not differ between monoculture and co-culture. Supernatants that had inhibitory effects on the two target organisms on average reduced their growth almost by half (Fig. 3). The maximal inhibitory effect observed even led to a four to five times reduced growth of *E. coli* WA321. Co-cultures that had a growth-promoting effect increased the growth of the target organisms by a factor of 1.5; however, in one case more than three-fold growth increase was observed for *E. coli* WA321 (Fig. 3).

Identification of key players within interactions

We further investigated whether specific taxonomic groups were capable of conferring growth-inhibiting or -promoting effects by interaction with other organisms in the co-cultures. Hence, we split the 1402 co-cultures tested based on the taxonomic classification of the two organisms involved.

On phylum level, inhibiting effects on growth were mostly observed for members of the Proteobacteria (Fig. 4). Co-cultures including Gammaproteobacteria revealed inhibiting effects for both *S. aureus* 533R4 and *E. coli* WA321 ($P < 0.001$ and $P < 0.001$, respectively). This equally applied to Betaproteobacteria for *S. aureus* 533R4 ($P < 0.05$) and to Alphaproteobacteria for *E. coli* WA321 ($P < 0.01$). In particular, co-cultures of Alphaproteobacteria and Betaproteobacteria had a significantly increased frequency of growth-inhibiting effects on both target organisms (*S. aureus* 533R4: $P < 0.01$; *E. coli* WA321: $P < 0.01$; Fig. 4).

On family level, mainly co-cultures between Pseudomonadaceae isolates showed a significantly increased growth inhibition frequency (*S. aureus* 533R4: $P < 0.05$; *E. coli* WA321: $P < 0.001$). Supernatants derived from co-cultures between Caulobacteraceae and Pseudomonadaceae isolates ($P < 0.001$), Oxalobacteraceae and Pseudomonadaceae ($P < 0.05$) as well as co-cultures between Flavobacteriaceae and Pseudomonadaceae isolates ($P < 0.01$) led to growth-inhibitory effects on *E. coli* WA321; co-cultures between Caulobacteraceae and Comomonadaceae ($P < 0.01$) as well as Pseudomonadaceae ($P < 0.05$) significantly inhibited the growth of *S. aureus* 533R4.

The growth of *S. aureus* 533R4 was not significantly promoted by any of the tested phylum–phylum interactions (Fig. 4A), whereas the growth of *E. coli* WA321 was promoted mostly by co-cultures between Bacteroidetes and Firmicutes ($P < 0.01$). Within these phyla, especially co-cultures between Sphingobacteriaceae and Paenibacillaceae led to a more frequent growth promotion ($P < 0.05$).

DISCUSSION

The exploration of new antimicrobial substances is of wide interest, not only in the context of discovery of novel compounds, but also to understand microbial interactions in various ecosystems. Classical techniques to screen for antimicrobial production have been carried out on solid agar plates using an overlay assay combined with visual screening for zones of inhibition (Tyc *et al.* 2014; Balouiri, Sadiki and Ibnsouda 2016) and thus, no analysis of growth-promoting effects was possible. Furthermore, the evaluation of these screenings is time consuming and strongly dependent upon a subjective evaluation. The newly developed high-throughput screening applied in this study is a fast and highly standardized method to detect growth-inhibiting as well as growth-promoting effects induced during monocultivation and co-cultivation of bacteria in liquid medium. Thereby, it provides new insights into microbial interactions in groundwater and opens up a new door to interaction studies in other environments.

The bacterial isolates obtained in this study represent up to 2.1% of the bacterial community of the oxic aquifer, which is high compared to the usual 0.25% of the microorganisms that are assumed to be culturable from freshwater environments (Amann, Ludwig and Schleifer 1995). However, a high percentage of phylogenetic groups of groundwater microbes lack cultured representatives. This is especially true for Candidate Phyla, such as the superphylum Cand. Parcubacteria, which constitute up to 50% of the microbial community in the groundwater of the pristine limestone aquifers and other groundwater ecosystems (Luef *et al.* 2015; Schwab *et al.* 2017). With their reduced genomes of around 1 Mbp (Luef *et al.* 2015) and reduced metabolic capacities, it is believed that their dependence on interaction explains why these microbes could not be cultivated in monocultures. Thus, our cultivation approach can only provide a limited view of microbial interactions occurring in groundwater.

Inhibitory effects dominated in both monocultures (6% promotion versus 38% inhibition) and co-cultures (3% promotion versus 13% inhibition) for both the target organisms used in this study. This suggests that interactions between groundwater bacteria are more antagonistic in nature than mutualistic.

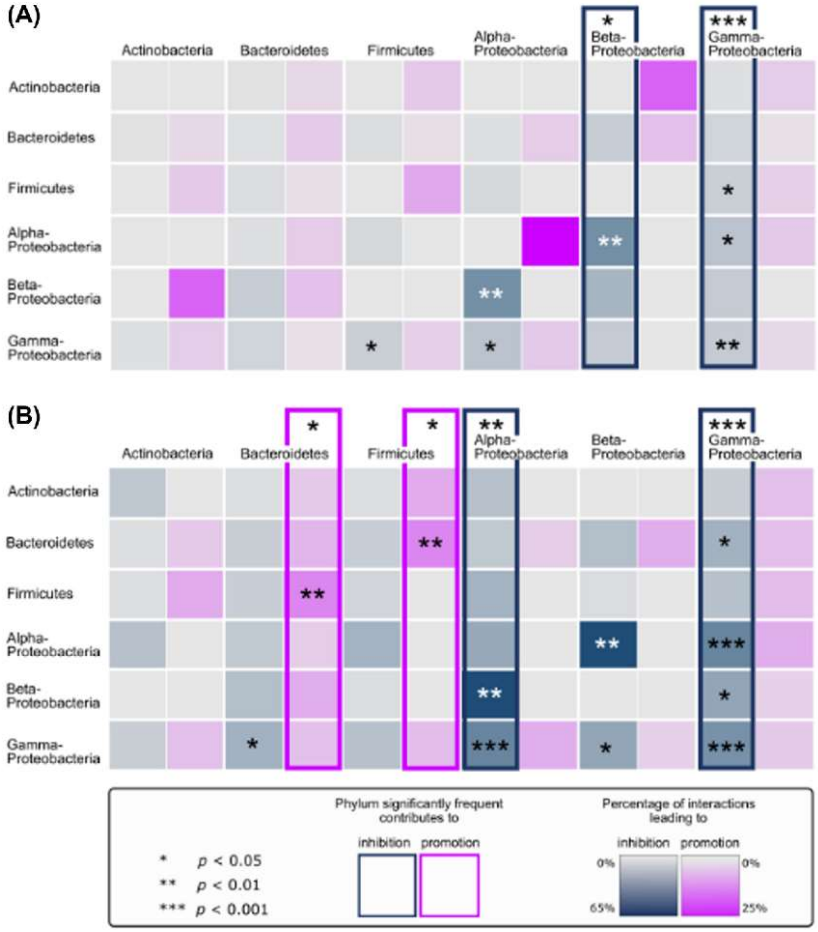

Figure 4. Heat map showing growth-inhibiting (blue shades) and -promoting (purple shades) effects of cell-free supernatants derived from co-cultures on phylum level on (A) *S. aureus* 533R4 and (B) *E. coli* WA321. *P*-values derived from Fisher's Exact Ratio test are indicated by asterisks, phyla that show an overall higher frequency of growth promotion or inhibition are highlighted through colored boxes.

Overall, co-cultivation of groundwater bacteria did not increase their potential to affect the growth of the tested target organisms, although almost half of the tested bacteria showed an effect in monoculture. Only growth promotion on *S. aureus* 533R4 increased from 0.7% to 2% in co-cultures, whereas growth promotion on *E. coli* WA321 and growth inhibition on both model organisms decreased by 50%–75%. The limited effect of co-cultures on growth affecting activities might be caused by the competitive situation for nutrients inside a co-culture, which prevents bacteria from investing in the production of secondary metabolites. In contrast to Cand. Parcubacteria that rely on metabolic interactions due to their reduced genomes (Luef *et al.* 2015), groundwater bacteria like the isolates tested in this study might not activate their full secondary metabolite potential in the presence of other microorganisms but rather invest on efficient uptake mechanisms for nutrients.

Limited growth-affecting activities of groundwater isolates incubated in co-culture are in disagreement with previous findings obtained with soil bacterial isolates (Garbeva *et al.* 2011; Traxler *et al.* 2013; Kinkel *et al.* 2014; Tyc *et al.* 2014). A high percentage of soil bacteria reveals antimicrobial activity only when tested in interactions (Tyc *et al.* 2014). In soil ecosystems, microorganisms are present in spatial proximity, whereas groundwater bacteria barely have physical contact with other bacteria. As exchange of secondary metabolites is dependent upon diffusion to the partner organism, this strategy might not be effective in pristine groundwater with very low microbial abundance (Opitz *et al.* 2014).

Nonetheless, our screening revealed that the groundwater microbiome seems to harbor a potential to produce secondary metabolites of both growth-promoting and -inhibiting effects. On the phyla level, growth-promoting effects were mostly conferred by bacteria belonging to the phyla Bacteroidetes and Firmicutes. In particular, supernatants derived from bacteria belonging to the families Sphingobacteriaceae and Paenibacillaceae showed growth-promoting effects on the used target organisms. These families are known producers of diverse secondary metabolites such as nonribosomal peptide synthetases (NRPs), lipopeptides, polyketide synthases (PKSs), PKS–NRPS hybrid compounds and others (Pawlowski *et al.* 2017; Shen *et al.* 2017), though a growth-stimulating effect has not yet been described. Proteobacteria, especially Pseudomonadaceae, well-known producers of antimicrobials (Raaijmakers, Weller and Thomashow 1997; de Bruijn *et al.* 2007; Matthijs *et al.* 2007; Silby *et al.* 2011), were key players in interactions with inhibitory effects on the target organisms. In our pristine groundwater

habitat bacteria belonging to the family Pseudomonadaceae make up to 1.5% of the total microbial community (Schwab *et al.* 2017) and could be therefore key players in interactions.

The complexity of microbial interactions in numerous environments has been poorly investigated and has so far often been focusing on the identification of antimicrobial substances. With our high-throughput screening method, growth-promoting effects can also be quantified, which can be applied to improve our understanding of microbial interactions in diverse environments. Studies on the microbiomes of different environments has increased exponentially during the last decade, revealing the tremendous diversity and complexity of microbial communities. At the same time, our knowledge about metabolic interactions that shape microbial communities is still rudimentary. More empirical work is required to understand microbial metabolic interactions of different environmental origins and high-throughput methods, such as the one described in this study, might be very useful.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflicts of interest. None declared.

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3. Predominance of *Cand.* Patescibacteria in groundwater is caused by their preferential mobilization from soils and flourishing under oligotrophic conditions

Martina Herrmann, Carl-Eric Wegner, Martin Taubert, **Patricia Geesink**, Katharina Lehmann, Lijuan Yan, Robert Lehmann, Kai Uwe Totsche and Kirsten Küsel

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Despite the widely observed predominance of *Cand.* Patescibacteria in subsurface communities, their input source and ecophysiology are poorly understood. Here we study mechanisms of the formation of a groundwater microbiome and the subsequent differentiation of *Cand.* Patescibacteria. In the Hainich Critical Zone Exploratory, Germany, we trace the input of microorganisms from forested soils of preferential recharge areas through fractured aquifers along a 5.4 km hillslope well transect. *Cand.* Patescibacteria were preferentially mobilized from soils and constituted 66 % of species-level OTUs shared between seepage and shallow groundwater. These OTUs, mostly related to *Cand.* Kaiserbacteraceae, *Cand.* Nomurabacteraceae, and unclassified UBA9983 at the family level, represented a relative abundance of 71.4 % of the *Cand.* Patescibacteria community at the shallowest groundwater well, and still 44.4 % at the end of the transect. Several *Cand.* Patescibacteria subclass-level groups exhibited preferences for different conditions in the two aquifer assemblages investigated: *Cand.* Kaiserbacteraceae surprisingly showed positive correlations with oxygen concentrations, while *Cand.* Nomurabacteraceae were negatively correlated. Co-occurrence network analysis revealed a central role of *Cand.* Patescibacteria in the groundwater microbial communities and pointed to potential associations with specific organisms, including abundant autotrophic taxa involved in nitrogen, sulfur and iron cycling. Strong associations among *Cand.* Patescibacteria themselves further suggested that for many groups within this phylum, distribution was mainly driven by conditions commonly supporting a fermentative life style without direct dependence on specific hosts. We propose that import from soil, and community differentiation driven by hydrochemical conditions, including the availability of organic resources and potential hosts, determine the success of *Cand.* Patescibacteria in groundwater environments.

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Predominance of *Cand.* Patescibacteria in Groundwater Is Caused by Their Preferential Mobilization From Soils and Flourishing Under Oligotrophic Conditions

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Despite the widely observed predominance of *Cand*. Patescibacteria in subsurface communities, their input source and ecophysiology are poorly understood. Here we study mechanisms of the formation of a groundwater microbiome and the subsequent differentiation of *Cand*. Patescibacteria. In the Hainich Critical Zone Exploratory, Germany, we trace the input of microorganisms from forested soils of preferential recharge areas through fractured aquifers along a 5.4 km hillslope well transect. *Cand*. Patescibacteria were preferentially mobilized from soils and constituted 66% of species-level OTUs shared between seepage and shallow groundwater. These OTUs, mostly related to *Cand*. Kaiserbacteraceae, *Cand*. Nomurabacteraceae, and unclassified UBA9983 at the family level, represented a relative abundance of 71.4% of the *Cand*. Patescibacteria community at the shallowest groundwater well, and still 44.4% at the end of the transect. Several *Cand*. Patescibacteria subclass-level groups exhibited preferences for different conditions in the two aquifer assemblages investigated: *Cand*. Kaiserbacteraceae surprisingly showed positive correlations with oxygen concentrations, while *Cand*. Nomurabacteraceae were negatively correlated. Co-occurrence network analysis revealed a central role of *Cand*. Patescibacteria in the groundwater microbial communities and pointed to potential associations with specific organisms, including abundant autotrophic taxa involved in nitrogen, sulfur and iron cycling. Strong associations among *Cand*. Patescibacteria themselves further suggested that for many groups within this phylum, distribution was mainly driven by conditions commonly supporting a fermentative life style without direct dependence on specific hosts. We propose that import from soil, and community differentiation driven by hydrochemical conditions, including the availability of organic resources and potential hosts, determine the success of *Cand*. Patescibacteria in groundwater environments.

Keywords: shallow subsurface, ultra-small bacteria, oligotrophy, community assembly, co-occurrence, *Cand.* Patescibacteria, *Cand.* Paceibacteria

INTRODUCTION

In recent years, the bacterial tree of life underwent a tremendous expansion through the discovery of the immense microbial diversity within the 'candidate phyla radiation' (CPR) (Hug et al., 2016). Recent phylogenetic and taxonomic analyses suggested the reclassification of the CPR as a single phylum, *Cand.* Patescibacteria (Parks et al., 2018), with 14 classes known so far. The majority of these taxa were predicted based on metagenomic analysis of habitats difficult to access, such as groundwater, deep sea sediments, permafrost, and the continental deep subsurface (Brown et al., 2015; Luef et al., 2015; Frey et al., 2016; Hubalek et al., 2016; Léon-Zayas et al., 2017). In fact, groundwater environments have turned out to contain a particularly high abundance of *Cand.* Patescibacteria, up to 38% of the total microbiomes (Bruno et al., 2017; Kumar et al., 2017; Schwab et al., 2017). As available information about these organisms is derived almost exclusively from (meta)genomic analyses, research needs to be aimed at elucidating their origin and ecophysiology to understand their success in these habitats.

Seventy-five to eighty-four percent of the cells in groundwater environments were previously found to be so-called low-nucleic acid (LNA) content bacteria as defined by flow cytometry, usually corresponding to cells smaller than 0.4 μm (Besmer et al., 2016; Proctor et al., 2018). Members of *Cand.* Patescibacteria in groundwater are especially abundant in the ultra-small fraction of cells (Miyoshi et al., 2005; Luef et al., 2015), i.e., cells that even pass through 0.2 μm pore size filters (Rappé et al., 2002; Wurch et al., 2016; Castelle et al., 2018). The widespread use of such filters for biomass collection likely contributed to the oversight of these microorganisms in past studies. In oligotrophic habitats like pristine groundwater, ultra-small cell size is thought to be evolutionarily advantageous, as the increased surface-to-volume ratio optimizes uptake of the sparse nutrients (Sowell et al., 2009). Lack of nutrients alone might further lead to a reduction of cell size in starving microorganisms (Hood and Macdonell, 1987; Vybiral et al., 1999; Young, 2006). Typically associated with inherently small cell sizes is a reduction in genome size by loss of expendable genes, which leads to a lower metabolic cost of reproduction (Giovannoni et al., 2014). Taken to the extreme, this can result in the loss of essential metabolic functions, which inevitably leads to dependencies on other organisms. Members of *Cand.* Patescibacteria, e.g., *Cand.* Paceibacteria or *Cand.* Microgenomatia, often show such reduced genomes of approximately 1 Mbp, and a lack of functional genes essential for amino acid or nucleotide biosynthesis (Brown et al., 2015), and hence, a host-dependent lifestyle of these organisms has been suggested (Hug et al., 2015; Castelle et al., 2018). Therefore, abundance and community structure of *Cand.* Patescibacteria in groundwater might not just be dependent on the ambient hydrochemical conditions, but also the availability of partners might shape their distribution patterns.

Despite the widely observed predominance of *Cand.* Patescibacteria in subsurface microbial communities, surprisingly little attention has been paid to the origin of these organisms and to the mechanisms by which they are introduced into groundwater and ultimately become the dominant members of the groundwater microbiome. In this work, we aimed to identify potential sources of *Cand.* Patescibacteria in groundwater, and assess key factors underlying their establishment and differentiation, focusing on groundwater hydrochemical conditions as well as potential interactions with other members of the groundwater microbial community as inferred from co-occurrence networks. We traced members of *Cand.* Patescibacteria along a 5.4 km soil and groundwater monitoring transect in the hillslope terrain of the Hainich Critical Zone Exploratory (CZE), a unique field site which allowed us to study the formation of the groundwater microbiome in the common geologic setting of thin-bedded mixed carbonate-siliciclastic bedrock (Küsel et al., 2016). We accessed soil seepage of the forested surface-recharge area, and upper slope shallow perched groundwater at 5 m depth down to downslope resources in fractured bedrock strata at about 90 m below the surface. Our results suggest the soils as the origin of *Cand.* Patescibacteria, as these organisms are readily mobilized with seepage and constitute the largest fraction of taxa shared between seepage and shallow groundwater. Within the groundwater, divergent trends in the preference for several hydrochemical parameters resulted in the differentiation of *Cand.* Patescibacteria communities across the two aquifer assemblages of our study site. Co-occurrence networks pointed to potential interactions with other bacterial groups, including autotrophs. However, the distribution patterns of various *Cand.* Patescibacteria groups appeared to be independent of specific partner organisms.

MATERIALS AND METHODS

Study Site, Sampling, and Chemical Analysis

Soil materials, seepage, and groundwater were collected from the Hainich CZE located in Thuringia, Germany, which was established in the framework of the Collaborative Research Center AquaDiva (Küsel et al., 2016). The location, geological setting, construction procedures as well as materials of groundwater wells were described elsewhere in detail (Küsel et al., 2016; Kohlhepp et al., 2017; Lazar et al., 2019). Outcropping bedrocks and aquifer strata of the hillslope terrain belong to the lithostratigraphic subgroup Upper Muschelkalk of the Germanic Triassic (Kohlhepp et al., 2017). The sloping strata of thin-bedded marine limestone-mudstone alternations host a multi-story aquifer system of a local groundwater flow system. Two aquifer assemblages were sampled: the limestone-dominated, karstified lower aquifer assemblage (HTL) and the mudstone-dominated upper aquifer assemblage (HTU) (Küsel et al., 2016; **Figure 1**). To follow the vertical transfer of microorganisms from soils (Chromic Cambisol; Cambisol) via seepage, we utilized tension-supported lysimeters (METER Group AG, Munich, Germany) installed in duplicates in 30 cm depth at a hilltop monitoring plot representing the forested preferential surface-recharge area of the sloping strata (Kohlhepp et al., 2017) (H1L1-1, H1L1-2; H1L3-1, H1L3-2: managed forest; H2L1-1, H2L1-2: unmanaged forest). The lysimeters were composed of a stainless-steel ring (diameter: 30 cm; height:

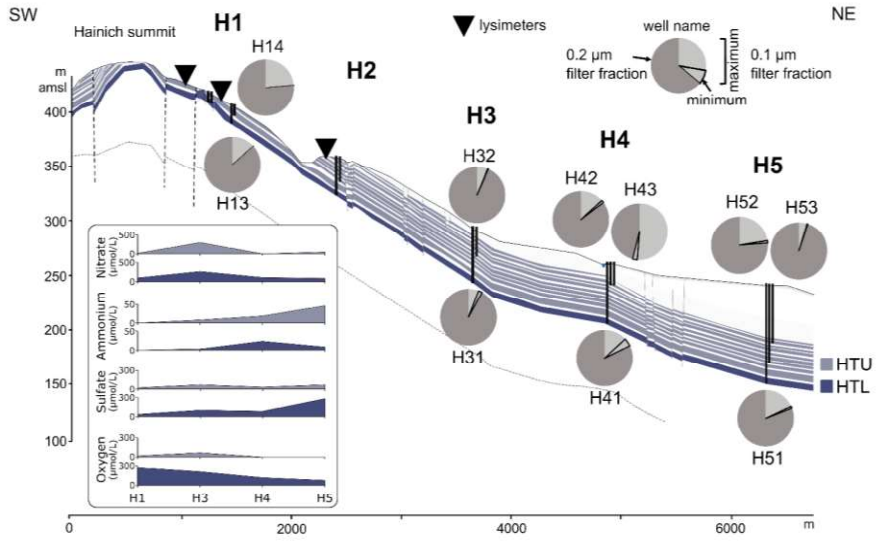

FIGURE 1 | Proportions of ultra-small cells in 0.1 and 0.2 µm pore size filter fractions from groundwater samples along the eastern Hainich hillslope, aquifer configuration, and selected physicochemical parameters. Groundwater wells are shown as vertical black lines. The cross section [from Kohlhepp et al. (2017), modified] shows the karstified main aquifer [HTL; (wells H13, H31, H41, H51)] that is characterized by massive limestone beds and higher surface-connection by hilltop preferential recharge areas. The hanging thin-bedded alternating limestone-mudstone strata that host the multi-story upper aquifer assemblage (HTU; wells H14, H32, H42, H43, H52, H53) exhibit lower hydraulic conductivities due to a lesser intensity of karstification, yielding longer groundwater residence times. Faults are indicated by vertical dashed lines. Lysimeter installations used in this study are shown as inverted triangles. Quantification was based on 16S rRNA gene targeted qPCR (H13, H14: one sampling time point; other wells: two (H31) or six sampling time points). Dark gray section of the pie chart represents proportion of bacterial 16S rRNA genes associated with the 0.2 µm filter fraction. Light gray section of the pie chart represents the maximum (encircled area: minimum) proportion of bacterial 16S rRNA genes associated with the 0.1 µm filter fraction.

10 cm) filled with glass beads (size/diameter: ~2 mm) to support and hydraulically connect the overburden, undisturbed soil, along with a porous silicon carbide suction plate (SIC320; pore size of ~20 µm; same manufacturer) at the bottom. Suction was applied via a battery powered vacuum controller (VS twin, same manufacturer) and regulated according to the prevailing soil matrix potential, measured constantly with a tensiometer (T8, same manufacturer).

For hillslope shallow groundwaters (ten wells) and recharge areas (3 plots) six to eight sampling time points were integrated (see **Supplementary Material**), complemented with a one-time sampling of forest top soil (TS; 10 cm depth) in five spatial replicates (samples H1-TS1 – H1-TS5, H2a-TS1 – H2a-TS5) in vicinity to the lysimeter installations at two locations (H1L1, H2L1) in September 2016 (n = 10). Soil samples were obtained using a sterile spatula, transferred to sterile 50 ml tubes, frozen on dry ice, and stored at −80°C until nucleic acid extraction. Number of temporal replicates differed across groundwater wells and lysimeters because not all the sampling sites yielded enough water for analysis at all six time points. Regular sampling of the groundwater and chemical analyses were described elsewhere (Küsel et al., 2016; Kohlhepp et al., 2017). Groundwater samples for molecular analysis were obtained in September, November, and December 2015 and in June, August, and November 2016 and were collected in autoclaved 10 L FLPE (fluorinated polyethylen) containers and kept at 4°C until filtration was performed within 1 h. Groundwater samples were filtered through sterile 0.2 µm polycarbonate filters (Nuclepore, Whatman), and the filtrate was collected and subsequently filtered through sterile 0.1 µm polycarbonate filters. The filtered volumes ranged from 6 to 20 L. Lysimeter samples were obtained at eight time points between November 2016 and March 2017 and were filtered through sterile 0.2 µm pore size and subsequently through sterile 0.1 µm pore size polyethersulfone (PES) filters (Supor, Pall Corporation), with 100–400 ml collected on one filter. All filters were stored at −80°C until nucleic acid extraction was performed.

Nanoparticle Tracking Analysis

Measurements of the size distribution and concentration of mobile particles in groundwater samples were performed by nanoparticle tracking analysis (NTA) using an NS500 instrument (NanoSight; Malvern Instruments Ltd., Worcestershire, United Kingdom), equipped with a light source (diode laser, 405 nm, power <60 mW), a high-sensitive CMOS-camera system, and video analysis software.

DNA Extraction, Quantitative PCR, and Amplicon Sequencing

DNA was extracted from soil, groundwater, and seepage filter retentates using the PowerSoil DNA Isolation Kit (MO BIO

Laboratories, CA, United States) following the manufacturer's protocol. Abundances of bacterial 16S rRNA genes were determined by quantitative PCR (qPCR) on a Mx3000P instrument (Agilent, Böblingen, Germany) using Maxima SYBR Green Mastermix (Thermo Fisher Scientific, Germany) and the primer combinations Bac8Fmod/Bac338Rabc (Daims et al., 1999; Loy et al., 2002) following cycling conditions previously described (Herrmann et al., 2012). Amplicon sequencing of bacterial 16S rRNA genes was carried out using the primer combination Bakt_341F/Bakt_805R (Herlemann et al., 2011). Generation of barcoded amplicons and amplicon sequencing using the Illumina MiSeq platform and V3 Chemistry (Illumina) was performed by LGC Genomics (Berlin, Germany) as previously described (Kumar et al., 2017). Sequence analysis of bacterial 16S rRNA amplicons was performed using Mothur (v.1.39.1) (Schloss et al., 2009), following the Mothur MiSeq SOP (Kozich et al., 2013) along with the SILVA bacteria reference alignment v132 (Quast et al., 2013) as previously described (Kumar et al., 2017). To implement the genome-based phylogeny recently proposed by Parks et al. (2018), resulting high quality bacterial 16S rRNA sequence reads were subsequently classified against the 16S rRNA reference database of the Genome Taxonomy Database (GTDB release 03-RS86, reference file bac_ssu_r86.1_20180911, provided at the GTDB website[1]). For all analyses that used sequence information from the total community, that is, merged information from the 0.1 and 0.2 µm filter fraction, we used 16S rRNA gene qPCR data to calculate how much each filter fraction contributed to the total community for a given sample. In the next step, we multiplied relative abundances of taxonomic groups in each filter fraction with these correction factors and used the sum of the corrected relative abundances in the 0.1 and 0.2 µm filter fraction as relative abundance of a given taxonomic group within the total community.

Sequence data obtained in this study have been deposited in the European Sequence Archive (ENA; accession numbers ERS2221375-ERS2221502 in bioproject PRJEB25133).

Estimation of Mechanisms of Community Assembly

The relative importance of deterministic selection vs. stochastic processes on bacterial community assembly was evaluated by pairwise community comparison based on the turnover in phylogenetic community composition and species composition using a null model approach according to Stegen et al. (2013, 2015). The R code for this analysis was provided by Stegen et al. (2013). This approach assumes that species of more close phylogenetic relationships share more similar ecological niches. The abundance-weighted β-mean-nearest taxa distance (βMNTD) was computed to evaluate the pairwise phylogenetic turnover between a given pair of communities with the R package picante (Kembel et al., 2010). The null distribution of βMNTD values was generated via 999 times of randomization under the null hypothesis that the bacterial communities have identical phylogenetic composition. During each randomization, the species names were moved randomly across the tips of the phylogeny and one βMNTD was calculated. β-nearest taxon index (βNTI) was calculated to represent the difference between observed βMNTD and the mean of the null distribution in standard deviation units. β-Nearest Taxon Index (βNTI) infers the relative importance of selection (βNTI > 2: variable selection; βNTI < −2: homogeneous selection) and stochasticity (| βNTI| < 2) in bacterial community assembly. To further characterize the mechanisms that underlie the stochastic processes in the bacterial community assembly, the re-scaled Raup-Crick probability index RC_{bray} (Chase et al., 2011) was calculated based on Bray-Curtis distance. The null distribution of RC_{bray} values was generated via 999 times of randomization under the null hypothesis that the bacterial communities have identical species composition. When the environmental selection is low (| βNTI| < 2), an RC_{bray} value less than −0.95 or over 0.95 indicates that homogenizing dispersal or dispersal limitation is the dominant assembly process, respectively. An RC_{bray} value between −0.95 and 0.95 suggests no dominant assembly process.

Calculations integrated data from the connected wells (H13, H31, H41 and H51 in the lower aquifer assemblage) taken in August 2016. Prior to analysis, data sets were subsampled to 7876 reads per sample, and the sequence information from the 0.1 and 0.2 µm filter fraction was merged for each groundwater well and time point. OTUs with low read numbers were retained in the data set. The phylogenetic tree was generated from the aligned sequences of the representative OTUs in Mothur based on the relaxed neighbor-joining method (Evans et al., 2006). Calculations were carried out using the R code provided by the original authors at github[2].

Co-occurrence Network Analysis

Network analyses were carried out using the R software framework (v. 3.4.2) (R Core Team, 2014) and the packages Matrix (v. 1.2.3)[3], igraph (1.1.2) (Csárdi and Nepusz, 2006), and SpiecEasi (v. 0.1.2) (Kurtz et al., 2015) including respective dependencies. OTU abundance information from the 0.1 and 0.2 µm filter fractions was merged for each site and time point prior to analysis to perform network analysis on the total community. We further filtered out OTUs that were represented by less than 100 sequence reads over all datasets or that were not present in at least 30% of all datasets. This step was included to minimize the interference from OTUs in network analysis that are only present in few samples or at low abundance, and to reduce the computational load for the network construction. This decomplexed OTU table contained 854 out of the original 189600 OTUs. These 854 OTUs accounted for 68% of the total sequence reads obtained from groundwater. The decomplexed OTU table was subsequently subjected to co-occurrence network reconstruction using Meinshausen–Bühlmann neighborhood selection (Meinshausen and Bühlmann, 2006) as an inference model in SpiecEasi. The settings were as follows:

lambda.min.ratio=1e-2, nlambda=20, icov.select.params=list (rep.num=50). Edge confidence values representing edge stability

[1]http://gtdb.ecogenomic.org/

[2]https://github.com/stegen/Stegen_etal_ISME_2013

[3]https://cran.r-project.org/web/packages/Matrix/index.html

and reproducibility were calculated based on random re-sampling of the data using the model selection scheme StARS (Stability Approach to Regularization Selection) (Liu et al., 2010). An igraph network object for downstream analysis was subsequently created based on afore-mentioned edge confidence values. The network was filtered for positive interactions (defined as positive model coefficients) before being analyzed for inherent network clusters using the cluster_greedy function of igraph. High confidence edges (edge confidence > 0.5) were extracted, the network re-clustered, and individual clusters were inspected by subgraphing based on cluster members. Network characteristics were assessed by analyzing edge confidence frequencies as well as degree distributions.

Growth Experiments, Flow Cytometry, and Transmission Electron Microscopy

To test the effect of organic carbon availability on cell size, 26 heterotrophic bacterial groundwater isolates previously obtained on Reasoners2A medium (Reasoner and Geldreich, 1985) at 15°C in the dark and taxonomically characterized by 16S rRNA gene sequencing (**Supplementary Table 1**) were cultivated with different concentrations of organic carbon. The strains were pre-incubated for 2 days in a modified liquid R2A medium, containing 0.6 g/L $K_2HPO_4 \times 3H_2O$, 0.1 g/L $MgSO_4 \times 7H_2O$, 0.6 g/L sodium pyruvate, 1.0 g/L peptone, 1.0 g/L caseinhydrolysat, 1.0 g/L yeast extract, and 1.0 g/L dextrose; corresponding to 880.0 mg/L C_{org}, for 2 days. The cultures were then centrifuged, and the cell pellets were washed twice in sterile 1 M NaCl solution to remove residual medium prior to inoculation of the cultures of the main experiment. To observe the adjustment of cell size to different C_{org} concentrations, the medium described above was used (a) in its undiluted version and (b) with carbon sources diluted to 0.088 mg/L C_{org}. All cultures were set up with a volume of 40 mL in 50 mL Greiner BioOne Cultivation tubes. 1 g of sterile silica beads was added to every cultivation tube in order to detach all cells from the tube walls by vortexing prior to measuring cell size distribution by flow cytometry. Incubations were carried out at 15°C in the dark under constant agitation. A control was run along with all dilution levels of C_{org}. After 5 days of cultivation, cell size distributions were analyzed by flow cytometry. Of each culture, 990 µl were incubated with 10 µl of SYBR Green II (Invitrogen) for 10 min at room temperature in the dark. Analysis was performed in a CyFlow Cube 6 (Sysmex, Germany), measuring forward scatter (FSC, related to cell size) and green fluorescence emission measured at 530 ± 30 nm (FL1) using a 488 nm laser, to discriminate and enumerate bacterial cells. All cytometric analyses were evaluated on a logarithmic scale using the FCS Express 5 Flow Research Editions (DeNovo) software. Stained cells were visually distinguished from background by plotting the FL1 versus FSC signal of the negative controls. For further analyses only, events that were identified as cells were considered and the median of all FSC values in one sample was calculated within the software as a proxy of cell size within every sample. One isolate (hainich_200, *Flavobacterium* sp.) was selected for TEM analysis on a Zeiss CEM 902 A electron microscope (Carl Zeiss AG, Oberkochen, Germany). Cell material was fixed with 2.5% (v/v) glutaraldehyde in cacodylate buffer (100 mM, pH 7.4) for 2 h at room temperature. Fixed samples were subsequently washed three times with cacodylate buffer, and post-fixed with 1% osmium tetroxide in cacodylate buffer for 2 h at 20°C. Next, samples were dehydrated in an ascending ethanol series and stained with 2% (w/v) uranyl acetate in 50% (v/v) ethanol. The samples were embedded in Araldite resin (Plano, Wetzlar, Germany), ultrathin sections (70 nm thickness) were cut using an ultramicrotome Ultracut E (Reichert-Jung, Vienna, Austria), and mounted on Formvar-carbon coated 100 mesh grids (Quantifoil, Großlöbichau, Germany). Ultrathin sections were stained with lead nitrate for 10 min (Venable and Coggeshall, 1965) and examined in a Zeiss CEM 902 A electron microscope (Carl Zeiss AG, Oberkochen, Germany) and imaged using a TVIPS 1k Fast-Scan CCD-Camera (TVIPS, Munich, Germany).

Statistical Analysis

Correlations between chemical parameters and relative abundances of OTUs as well as taxonomic groups were assessed using Spearman rank correlation coefficients (two-sided) in PAST (Hammer et al., 2001). Differences of first-degree neighbors in co-occurrence networks were determined using Mann–Whitney *U* test in PAST.

RESULTS

Across all the groundwater wells of the Hainich CZE, organisms of the *Cand.* Patescibacteria represented the largest fraction of the groundwater microbial communities, with relative abundances ranging from 17 to 79%. *Cand.* Patescibacteria were especially enriched in the ultra-small fraction of cells: Following sequential filtration of groundwater through 0.2 and 0.1 µm pore size filters, up to 83% of the community collected on the 0.1 µm pore size filters were related to class *Cand.* Paceibacteria (groups previously referred to as *Cand.* Parcubacteria; Brown et al., 2015; Hug et al., 2016) of the phylum *Cand.* Patescibacteria (**Supplementary Figure 1A**). The eight most abundant taxa within the class *Cand.* Paceibacteria showed enrichment factors between 1.9- and 4.1-fold in relative abundance between the 0.2 and 0.1 µm pore size fraction (**Supplementary Figure 2**). Classes *Cand.* Microgenomatia and *Cand.* Saccharimonadia of the *Cand.* Patescibacteria likewise displayed a tendency to occur in the 0.1 µm filter fraction, with respective enrichment factors of 2.1 and 1.3. Conversely, members of classes ABY1 and *Cand.* Gracilibacteria, were strongly reduced in relative abundance in the ultra-small fraction, with enrichment factors of 0.7-fold to less than 0.0001-fold. To estimate how the population of a given taxonomic group was distributed across the 0.1 and 0.2 µm filter fraction, we combined qPCR-based information of bacterial abundances with sequencing data (**Supplementary Figure 3**). For several family- and order-level groups of *Cand.* Paceibacteria, between 15 and 30% of their total population were estimated to pass through the 0.2 µm filters.

The majority of microbial taxa with cultured representatives were predominantly found in the 0.2 µm filter fraction (**Supplementary Figures 2**, **3**), with the exception of spore forming *Firmicutes* and *Spirochaetota*, whose spiral shape at a diameter lower than 0.2 µm might allow them to pass through the filters. Consequently, well-studied microbial taxa such as *Proteobacteria*, *Nitrospirota*, *Planctomycetota*, *Bacteroidota*, and *Actinobacteriota* feature larger cell sizes, which agrees with previous flow-cytometry-based findings by Proctor et al. (2018). However, even members of these groups were occasionally detected in the 0.1 µm filter fraction. Using 26 heterotrophic bacterial strains of these taxa obtained from the Hainich CZE (**Supplementary Table 1**), we tested whether a reduction of cell size due to starvation in the oligotrophic groundwater could explain this phenomenon. In fact, 80% of these isolates showed reduced cell sizes based on the forward scatter (FSC) signal in flow cytometric analysis (**Figure 2**), when incubated for 5 days in conditions resembling the pristine groundwater in comparison to full medium with 880 mg L^{-1} C_{org}. Of note, one *Flavobacterium* isolate (hainich_200) showed a 96% decrease of the FSC signal in the starved cultures. Transmission electron microscopy confirmed a strong reduction of cell size, from 3.2 ± 2.0 µm to 0.98 ± 0.19 µm, along with a drastic change in cell shape (**Figure 2**). The isolates used were affiliated with bacterial genera which we had also detected in the groundwater bacterial communities based on 16S rRNA gene targeted amplicon sequencing. Relative abundances of the respective sequence reads suggested that these genera represented 6.9% of the total groundwater bacterial community. Hence, this wide-spread tendency for a reduction of cell size under nutrient limited conditions might explain why, apart from the dominant *Cand.* Patescibacteria, also classical heterotrophs were found in the ultra-small fraction.

The relative abundances of such ultra-small cells were surprisingly high, comprising up to 54% of the groundwater bacterial populations (**Figure 1** and **Supplementary Figure 4**), as determined by qPCR. Bacterial 16S rRNA gene abundances were $1.2 \times 10^7 - 8.6 \times 10^8$ genes L^{-1} groundwater for the 0.2 µm filter fraction and $3.3 \times 10^5 - 8.1 \times 10^7$ genes L^{-1} for the 0.1 µm filter fraction (**Supplementary Table 2**). Mean fractions of ultra-small cells in each well along the groundwater transect ranged from 2.0 to 19.7%. These estimates are rather conservative, given that some ultra-small cells will be retained on 0.2 µm filters, e.g., if they occur in aggregates or due to filter clogging at higher particle load. Similarly, ultra-small bacteria affiliated with *Cand.* Patescibacteria probably harbor only one 16S rRNA operon (Brown et al., 2015) while operon numbers may be in the range of one (*Cand.* Patescibacteria, *Thermodesulfovibrionia*, *Nitrospirota*, *Brocadiae*) or two to four (*Alpha-* and *Gammaproteobacteria*) for the bacteria commonly observed in the communities on the 0.2 µm filters [information derived from rrnDB-website[4] (Stoddard et al., 2015)]. These differences may additionally lead to an underestimation of the fraction of ultra-small cells.

Nanoparticle tracking analysis measurements, providing an unbiased picture of the particle load, in fact revealed that in the shallow groundwater at the hilltop position of our groundwater well transect, more than 60% of the particles were consistently smaller than 0.2 µm (**Figure 3**). The unique design of our monitoring wells, however, allows for the sampling of suspended particles up to five millimeters in size (Küsel et al., 2016) These particles, comprising not only organisms, but also inorganic

[4]https://rrndb.umms.med.umich.edu/

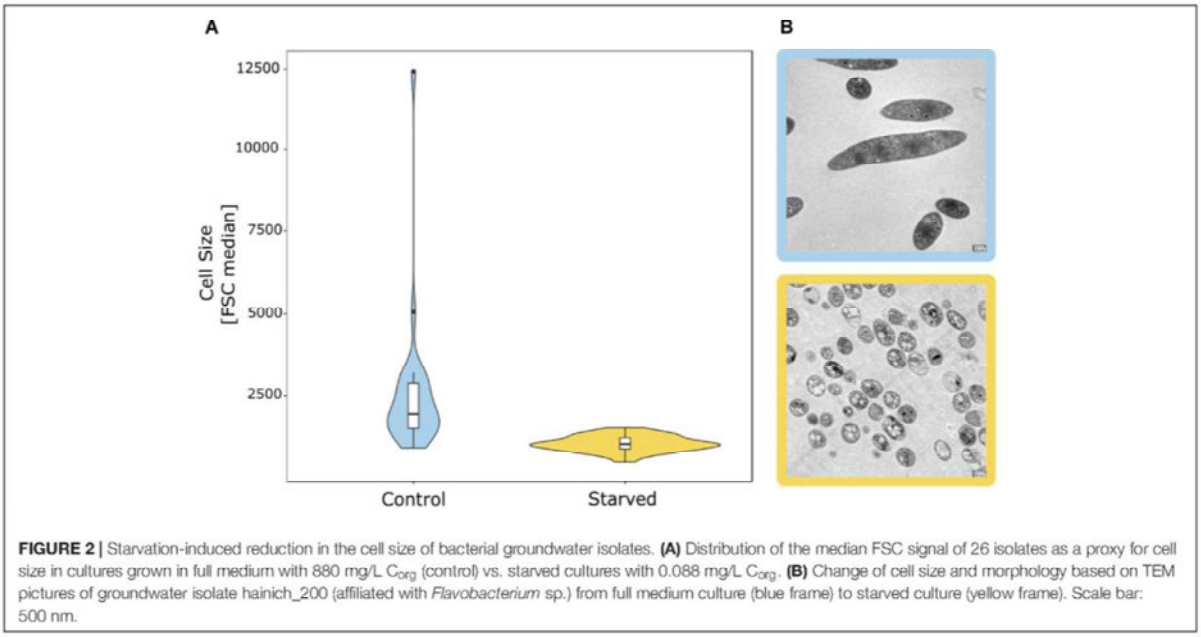

FIGURE 2 | Starvation-induced reduction in the cell size of bacterial groundwater isolates. **(A)** Distribution of the median FSC signal of 26 isolates as a proxy for cell size in cultures grown in full medium with 880 mg/L C_{org} (control) vs. starved cultures with 0.088 mg/L C_{org}. **(B)** Change of cell size and morphology based on TEM pictures of groundwater isolate hainich_200 (affiliated with *Flavobacterium* sp.) from full medium culture (blue frame) to starved culture (yellow frame). Scale bar: 500 nm.

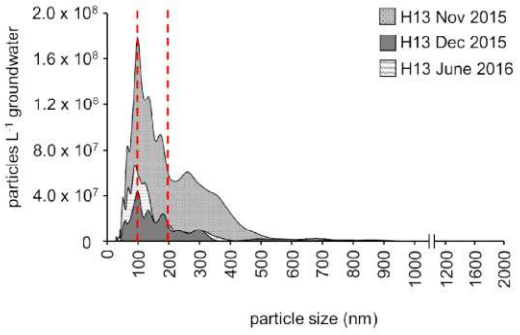

FIGURE 3 | Content and size distribution of groundwater particles from the first well of the transect, H13 (5.1 m below the surface), assessed by nanoparticle tracking analysis (NTA) for three different time points (November 2015, December 2015, January 2016; one replicate per time point). The red lines indicate filter pore size commonly used for microbial biomass collection in environmental surveys (0.2 μm) and the (0.1 μm) filter pore size additionally used here.

and organic material, are mobilized by infiltrating precipitation from soils through weathered rocks and into the groundwater. With 4.0×10^9 to 2.2×10^{10} particles per L in the well at the hilltop position, this translocation from surface to subsurface might be an important entry point for ultra-small cells of *Cand.* Patescibacteria into the groundwater. To identify the origin of *Cand.* Patescibacteria hence required us to trace back the flow of water to the soils of the preferential recharge area.

Cand. Patescibacteria Are Readily Mobilized From Soils

One great strength in the design of the Hainich CZE monitoring transect is the ability to follow the formation of the groundwater microbiome. Microbial populations can be traced from their potential origin, forest soils in the preferential surface-recharge area, vertically in seepage collected at 30 cm depth, down to upper slope shallow perched groundwater and downslope groundwater in the fractured limestone-mudstone alternations.

Surprisingly, members of *Cand.* Patescibacteria already dominated seepage from top soil horizons (30 cm depth), with relative abundances up to 50% (mean: 36 ± 12%, n = 10, **Figure 4A** and **Supplementary Figure 1**), although they represented only 0.55% (±0.34, n = 10) of the total bacterial community in forest soil. Likewise, abundances of 21 to 40% (mean: 30 ± 6%, n = 15) were observed throughout the lower aquifer assemblage. In addition to *Cand.* Patescibacteria, *Nitrospirota* became the most abundant group in the groundwater, although being only rarely present in soil and seepage (**Supplementary Figure 1**). *Acidobacteriota*, *Actinobacteriota*, and *Planctomycetota*, which made up more than 37% of the soil bacterial community, were either not mobilized into the seepage at all, or in low abundance, and their relative abundances decreased substantially in the groundwater (**Supplementary Figure 1A**). Enrichment factors in seepage compared to soil for *Alpha-* and *Gammaproteobacteria* were one to two orders of magnitude lower than for members of *Cand.* Patescibacteria. Nevertheless, they still formed a stable fraction in seepage and groundwater communities (**Figure 5** and **Supplementary Figure 1A**). Overall, 9.5% of the species-level OTUs in soil - assigned using a 97% sequence identity cut-off - were shared with seepage. The strong enrichment of *Cand.* Patescibacteria in the seepage compared to soil was confirmed when following species-level OTUs, revealing enrichment factors higher than 100 especially for OTUs affiliated with *Cand.* Nomurabacteraceae, *Cand.* Kaiserbacteraceae, and unclassified UBA9983 at the family level (**Supplementary Figure 5**). Altogether, these findings suggested a preferential mobilization and vertical transport of *Cand.* Patescibacteria from soils into the subsurface, confirming our assumptions that soils of the preferential recharge area are an important source of these organisms. In the next step, we aimed to elucidate how the mobilized organisms were thriving in the groundwater, and which parameters influenced their distribution patterns.

Differentiation of Groundwater *Cand.* Patescibacteria Communities

Along the sloping bedrock strata of the Hainich CZE, the groundwater microbiome can be sampled via several observation wells along a 5.4 km horizontal transect, from 5.1 m (well H13) down to 88 m (well H51) below the surface within a connected aquifer assemblage. The wells H13 to H51 represent an increase in lateral distance to the respective preferential recharge areas, assuming a higher surface-connectivity of the high-permeability strata of this aquifer assemblage that outcrop at uphill positions (Kohlhepp et al., 2017). Interestingly, *Cand.* Paceibacteria and candidate class ABY1, the dominant *Cand.* Patescibacteria classes, showed divergent distributions along the aquifer assemblage (**Figure 4B**). *Cand.* Paceibacteria consistently made up one to two thirds of the *Cand.* Patescibacteria community in soil and seepage, respectively, and about 50% in groundwater from well H13 to downstream positions (well H51). Candidate class ABY1, however, were barely detected in soil and seepage but increased continuously in relative abundance in groundwater with increasing distance to the hilltop preferential recharge area. In the deepest well, H51, candidate class ABY1 made up 25% of the *Cand.* Patescibacteria community. Similarly, we observed an increase in the fraction of *Cand.* Gracilibacteria from less than 1% in soil and seepage to 5.7% in well H13 and 10.8% in downstream well H51. However, not all *Cand.* Patescibacteria mobilized from soil were able to thrive in the groundwater. *Cand.* Saccharimonadia dominated in soil and were still abundant in seepage, but decreased continuously along the groundwater transect (**Figure 4B**). These organisms are able to metabolize sugar compounds under oxic and anoxic conditions and in association with plant tissue (Albertsen et al., 2013; Kindaichi et al., 2016; Beckers et al., 2017), and hence might be more adapted to soils or near-surface habitats.

To elucidate the mechanisms driving *Cand.* Patescibacteria distribution patterns, we analyzed the total microbial community structure from soil, seepage, and all groundwater wells based on species-level OTUs, confirming that selected OTUs can be traced

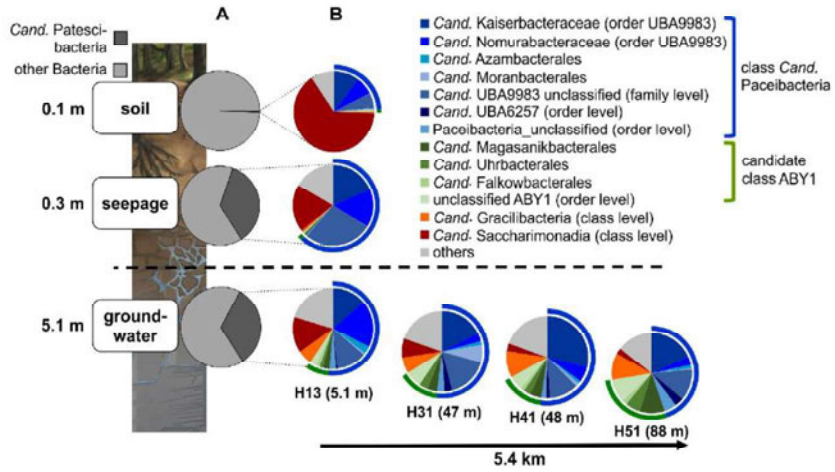

FIGURE 4 | Community structure of *Cand.* Patescibacteria in soil, seepage and groundwater. **(A)** Changes in the relative fraction of *Cand.* Patescibacteria within the bacterial communities from soil via seepage to groundwater and **(B)** changes in the order-level community structure of *Cand.* Patescibacteria (family level for *Cand.* Kaiserbacteraceae, *Cand.* Nomurabacteraceae and unclassified UBA9983; class level for *Cand.* Gracilibacteria and *Cand.* Saccharimonadia) from soil to seepage and groundwater and along the groundwater observation transect (lower aquifer assemblage). Data are based on 16S rRNA gene-targeted Illumina MiSeq amplicon sequencing and represent mean values of 10 spatial replicates for soil, mean values from five lysimeters with each one sampling time point except for H1L1-2 (n = 6) (total n = 11), and one (H13), two (H31) or six different sampling time points for groundwater (total n = 15). Seepage and groundwater samples integrate data from the 0.1 and 0.2 μm filter fractions.

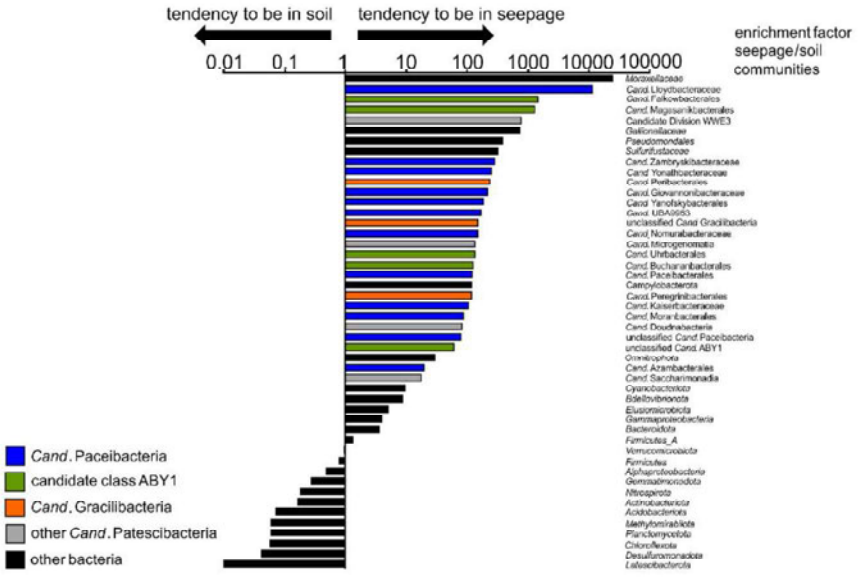

FIGURE 5 | Enrichment factors in seepage compared to soil for selected bacterial taxa (order/family level for *Cand.* Patescibacteria, class level for *Proteobacteria*, phylum level for other Bacteria). For calculation of these factors, mean values of relative abundances of a given taxon across all seepage samples from four locations and different time points (n = 10) were divided by mean values of relative abundances of the same taxon across all forest soil samples (n = 10). Relative abundances of taxa are based on 16S rRNA gene-targeted Illumina MiSeq amplicon sequencing.

from forest soil or forest seepage to the groundwater of the lower aquifer assemblage. Their relative abundances increased from less than 0.001% in forest soil or seepage to 4.6% in the groundwater of well H13 (Otu000184, *Cand.* Nomurabacteraceae) or to 6.9 and 2.8% in wells H41 and H51 (Otu000001, *Cand.* Kaiserbacteraceae) (**Supplementary Figure 6**). In contrast,

other soil-derived OTUs were also constantly detectable in the groundwater but at much lower relative abundances (Otu000012, *Cand.* Kaiserbacteraceae; Otu000014, *Cand.* Nomurabacteraceae). Groundwater at well H13 still shared about 35% of *Cand.* Patescibacteria-affiliated OTUs with seepage, while this fraction decreased to 6.6% at well H51 (**Supplementary Figure 7**). Seepage-derived OTUs contributed major parts of the *Cand.* Patescibacteria community, with 71.4% relative abundance at the hill top position (H13), contributing 23.5% to the total microbial community. Even at well H51, they formed 44.4% of the *Cand.* Patescibacteria community and 13.7% of the total microbial community (**Supplementary Figure 7**). In contrast, the shared fraction was lower between soil and groundwater (**Supplementary Figure 7**), probably due to the fact that only a low fraction of the soil OTUs was mobilized with seepage. Moreover, some potentially shared *Cand.* Patescibacteria OTUs may have been overlooked given the overall low fraction of *Cand.* Patescibacteria in the soil communities.

Following the models of Stegen et al. (2013, 2015), we estimated the contribution of different mechanisms to the formation of the groundwater microbiomes of the lower aquifer assemblage. Stochastic processes played a dominant role for bacterial community assembly, as indicated by β-nearest taxon indices between −0.61 and 1.63 for all pairwise comparisons between bacterial communities in the groundwater wells. The RC_{bray} values for all pairwise comparisons were 1, indicating that dispersal limitation, and hence impediment of the transport of microbes between wells, was the primary mechanism influencing community assembly. Due to the uniform hydrochemistry of the lower aquifer assemblage, community assembly was not driven by variable selection, i.e., the selective influence of environmental parameters on different microbial groups. The multi-story subsurface architecture of the Hainich CZE provides several distinct clusters of groundwater chemistry (Kohlhepp et al., 2017), from oxic to anoxic conditions, which support a high metabolic diversity of the groundwater microbial communities. Hence, we extended our analysis to both aquifer assemblages, to explore whether hydrochemical preferences were driving the differentiation of the *Cand.* Patescibacteria community.

Effect of Hydrochemical Parameters on *Cand.* Patescibacteria Community Differentiation

To identify their hydrochemical preferences, we correlated relative abundances of different *Cand.* Patescibacteria taxa with environmental parameters. Five distinct clusters of *Cand.* Patescibacteria taxa were observed based on the obtained correlations, showing no class-specific separation (**Figure 6**). These clusters showed widely contrasting preferences with regard to the concentration of nitrate and sulfate, as well as ammonium, sodium, potassium, and magnesium. Interestingly, we also observed positive correlations with oxygen concentration for *Cand.* Kaiserbacteraceae (*Cand.* Paceibacteria), *Cand.* Jacksonbacterales (ABY1) and further taxa, while *Cand.* Nomurabacteraceae (*Cand.* Paceibacteria), *Cand.* Komeilibacterales, and *Cand.* Magasanikibacterales (both ABY1) showed negative correlations with oxygen. Concentrations of TOC were usually less than 3 mg L^{-1} and showed only minor spatiotemporal variation across the two aquifer assemblages, yielding mostly non-significant correlations with the bacterial taxa included here (data not shown).

We further included autotrophic bacteria involved in key processes in our groundwater, like nitrification, sulfur and iron oxidation, and anammox (Herrmann et al., 2015; Kumar et al., 2017; Schwab et al., 2017) in our analysis. Interestingly, *Cand.* Magasanikibacterales showed similar correlation patterns to anammox-performing *Brocadiaceae*, iron-oxidizing *Gallionella* and thiosulfate-oxidizing *Sulfuricella*, while *Cand.* Kaiserbacteraceae shared hydrochemical preferences with ammonia-oxidizing *Nitrosospira*. These common preferences for environmental conditions indicate a co-localization of *Cand.* Patescibacteria and key autotrophs along the well transect. With their reduced genomes and limited biosynthetic capabilities, *Cand.* Patescibacteria depend on the uptake of nucleotides and amino acids from co-localized organisms (Brown et al., 2015). The presence of these autotrophs hence might be another factor driving the distribution patterns of *Cand.* Patescibacteria.

Support for a Central Role of *Cand.* Patescibacteria in Community Networks

To identify potential associations of *Cand.* Patescibacteria with specific bacterial taxa across the two aquifer assemblages, we performed co-occurrence network analysis. The high complexity of interactions present in the diverse groundwater microbial communities was reduced by restricting the analysis to 854 OTUs that were represented by at least 100 sequence reads across all samples. These OTUs represented 68% of the total sequence reads of all the groundwater samples. Moreover, we focused on positive correlations with edge confidence above a cutoff of 0.5 only (**Supplementary Figure 8**).

All seven distinct clusters of OTUs revealed by the network analysis contained members of *Cand.* Patescibacteria, pointing out their central role in the groundwater microbial communities (**Figure 7A**). Especially in clusters 1 and 7, *Cand.* Patescibacteria OTUs were dominant, constituting 40 and 70% of all OTUs, respectively (**Figures 7A,B**). The most abundant families in these clusters were *Cand.* Kaiserbacteraceae and *Cand.* Nomurabacteraceae of the order *Cand.* Paceibacteria. Compared to the other *Cand.* Patescibacteria classes, *Cand.* Paceibacteria co-occurred more often with *Cand.* Patescibacteria-affiliated OTUs. In contrast, first-degree neighbors of candidate class ABY1, *Cand.* Gracilibacteria, and *Cand.* Saccharimonadia included a significantly larger fraction of non-Patescibacteria OTUs (Mann–Whitney *U* test, $p = 0.0025$, **Supplementary Figure 9**).

As previous studies suggested an important role of autotrophic microorganisms in the groundwater of the Hainich CZE (Herrmann et al., 2015; Kumar et al., 2017; Nowak et al., 2017; Schwab et al., 2017), we were specifically interested in co-occurrence patterns that would suggest specific interactions between *Cand.* Patescibacteria and autotrophs. The putative nitrifiers, anammox bacteria, as well as iron and thiosulfate

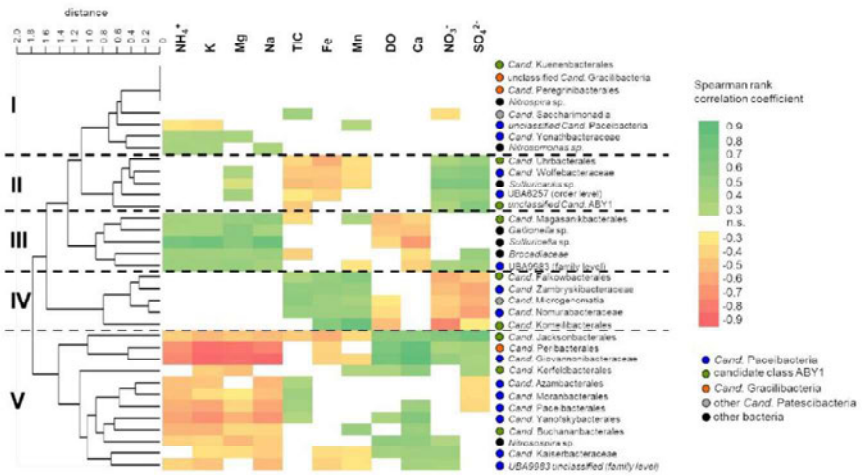

FIGURE 6 | Correlation of selected class-, order- and family- level bacterial groups with environmental parameters across the two aquifer assemblages. Correlations were calculated using Spearman rank correlation coefficients (r_S) with acceptance of significance at $p < 0.05$, $n = 46$. Colors indicate strength of correlation from $r_S = 0.9$ to $r_S = -0.9$. Taxa were clustered according to similar patterns in their correlation with environmental parameters using paired group algorithm (UPGMA) and Euclidean similarity index. DO, dissolved oxygen; TIC, total inorganic carbon.

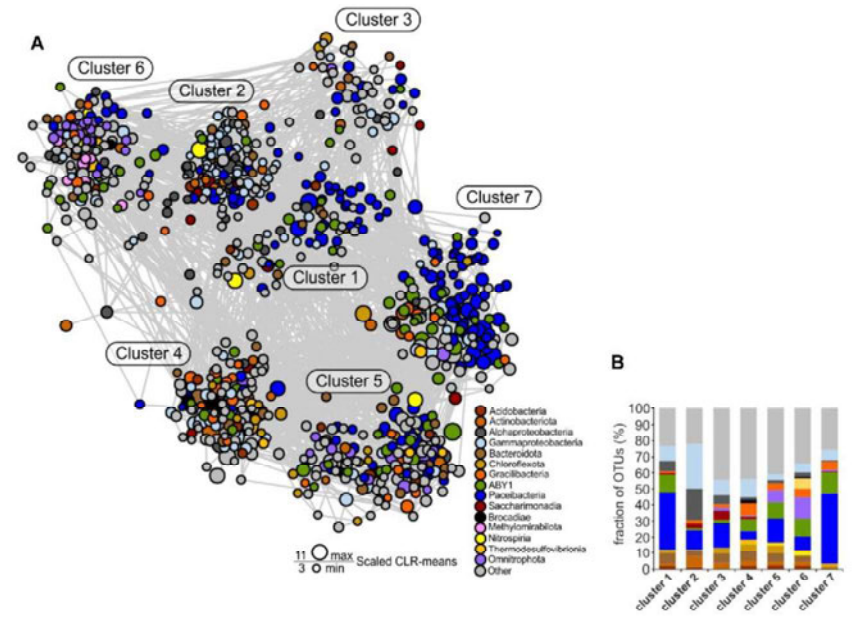

FIGURE 7 | Co-occurrence network of species-level OTUs across the two aquifer assemblages. For each sample, data sets from the 0.2 and 0.1 μm filter size fraction were merged. Only significant edges are displayed. **(A)** Co-occurrence network. Construction was based on a centered log-ratio (CLR) transformed OTU table and Meinshausen-Bühlmann neighborhood selection as graphical inference model. **(B)** Detailed view of the relative fraction of taxonomic groups across the seven clusters of the network.

oxidizers in the groundwater co-occurred more frequently with OTUs affiliated with candidate class ABY1 or *Cand.* Gracilibacteria than with *Cand.* Paceibacteria. First-degree neighbors of putative autotrophs included 22 and 35% of all ABY1 or *Cand.* Gracilibacteria-affiliated OTUs in the network but only 13% of *Cand.* Paceibacteria-affiliated OTUs.

The segregation of the seven clusters resulting from OTU co-occurrence patterns could only marginally be explained based on

the correlation of these OTUs with hydrochemical parameters (**Figure 8**; Spearman rank correlation coefficient, r_S, calculated individually for each OTU). Clusters 4 and 6 seemed to be partially driven by ammonium concentration (cluster 4: mean r_S calculated from all OTUs of that cluster = 0.50 ± 0.13) as well as oxygen and calcium concentrations (cluster 6: r_S (oxygen) = 0.37 ± 0.15; r_S (calcium) = 0.46 ± 0.12), but no further correlations driving the differentiation of other clusters were observed.

DISCUSSION

Microbiomes in subsurface habitats, from shallow aquifers to deep continental crystalline rocks, are typically characterized by high abundance of members of *Cand.* Patescibacteria and by ultra-small cell sizes (Luef et al., 2015; Hubalek et al., 2016; Proctor et al., 2018). So far, little is known about the formation of subsurface microbiomes and about the origin of *Cand.* Patescibacteria in groundwater. An autochtonous (syn-) sedimentary origin and long-term descent of these organisms in our marine bedrock (Middle Triassic) is unlikely, since rock core analyses of the Hainich CZE reveal neither endolithic *Cand.* Patescibacteria, nor their presence on rock fractures (Lazar et al., 2019). Alternatively, these organisms must be introduced from the surface and establish and maintain stable populations in the groundwater. The infrastructure of the Hainich CZE to sample soil, seepage, and groundwater in the topographic recharge and transit area of the local groundwater flow system allows for the analysis of the introduction of soil-derived microorganisms into the groundwater. Since the strata of the lower aquifer assemblage (HTL) crop out at upper hill areas that are thought to function as preferential surface-recharge areas (Kohlhepp et al., 2017), we followed changes in the groundwater microbiome along the subsurface water flow from its origin through shallow perched groundwater to deeper saturated zones. Seepage-dependent release and transport - including colloid-assisted transport - are passive dispersal mechanisms (Dibbern et al., 2014; Lehmann et al., 2018; Zhang et al., 2018) that link soil, subsoil and groundwater microbiomes, allowing organisms to colonize the groundwater and spread along the multi-story aquifer system of the Hainich CZE. This repetitive flow of water and transport of cells and matter creates a constant disturbance characteristic for shallow groundwater ecosystems, influencing community assembly. Soil-derived organisms are transported into the groundwater, where they become part of the community present, and can thrive and increase in abundance if encountering suitable conditions. In addition, interactions and exchange between planktonic groundwater microorganisms and those that are attached to aquifer rock may affect the assembly of groundwater microbial communities. In fact, recent investigations at our study site suggested considerable mobilization and (re)dispersal of attached microorganisms, as more than 44% of rock matrix-associated genera were also found in the groundwater (Lazar et al., 2019). Interestingly, *Cand.* Patescibacteria accounted for less than 1% of the rock matrix community (Lazar et al., 2019), suggesting that their distribution in the two aquifer assemblages might be less affected by interactions between planktonic and attached populations.

Here we show that *Cand.* Patescibacteria make up a large part of this microbial input into the groundwater. Forested soils in the hilltop preferential recharge area are the most probable source of *Cand.* Patescibacteria at our study site, as these organisms were mobilized in high abundance in seepage, despite their low relative abundance in the soil microbial communities. Likewise, Zhang et al. (2018) recently observed high abundance of *Cand.* Patescibacteria in seepage collected beneath maize-planted agricultural soils, indicating preferential mobilization as a common trait for these organisms. Surface charge of cells, hydrophobicity, or cell surface macromolecules

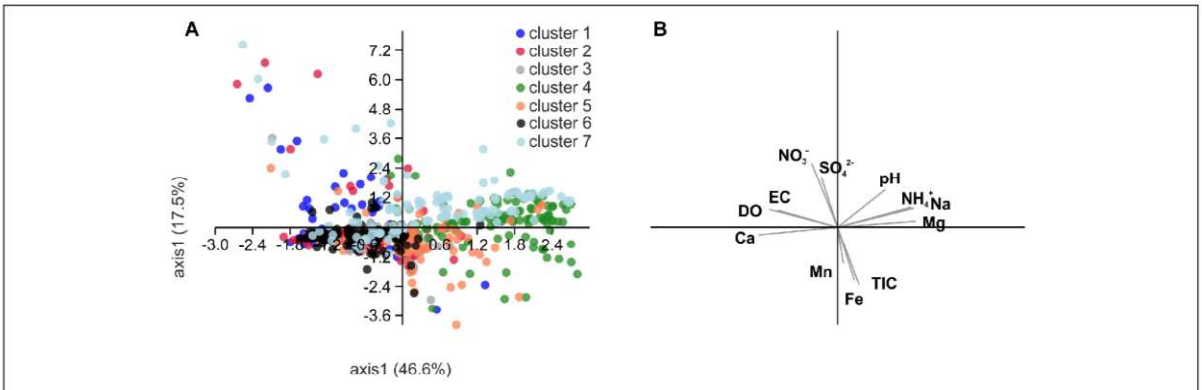

FIGURE 8 | Scatter plot derived from principal component analysis (PCA) of Spearman rank correlation coefficients of selected hydrochemical parameters with relative abundances of the OTUs included in the network of **Figure 7 (A)** and biplot of the correlated hydrochemical parameters **(B)**. To test for an effect of chemical parameters on the association of OTUs in the identified subclusters of the network, we performed pairwise correlations of relative abundances of each OTU with hydrochemical parameters and then subjected the resulting correlation coefficients of significant correlations to PCA. Correlation coefficients were calculated with relative abundances of OTUs from ten groundwater wells and one (H13, H14), two (H31) or six sampling time points per well (total n = 46). DO, dissolved oxygen; TIC, total inorganic carbon.

have been discussed as factors influencing transport of bacteria in porous media (Wan et al., 1994; Bolster et al., 2009; Kim et al., 2009). *Cand.* Patescibacteria encode large cell surface proteins, most likely involved in the attachment to other microorganisms (Castelle et al., 2018). However, a potential supporting effect of these surface proteins on the mobilization by percolating water in soils remains currently unclear. The fact that surface charge is negative for soil mineral particles and is most likely also negative for *Cand.* Patescibacteria cells, as it holds for most microorganisms (Koyama et al., 2013), could contribute to generally favorable conditions for cell dispersal in soil. The low ionic strengths observed in soil seepage (<2 mmol L^{-1}) reduce attachment (Wang et al., 2013), further promoting microbial dispersal. In the saturated zone, ionic strengths range from 5 (shallow perched groundwater) to ~20 mmol L^{-1} (deepest well), pointing to variable but also unfavorable conditions for the subsurface mobility of microbial cells. This reduced mobility, together with the heterogeneous structure of the fractured aquifer rock, might explain why the compositional turnover of microbial communities across the lower aquifer assemblage was dominated by dispersal limitation.

The Hainich CZE provides several distinct hydrochemical zones on a single hillslope, differing strongly in concentrations of oxygen, nitrogen compounds, iron, and sulfur compounds and hence supporting a high functional diversity of microorganisms. Consequently, when investigating the distribution patterns of *Cand.* Patescibacteria, we observed a high and taxon-specific variability both for correlations with environmental parameters and co-occurrence with putative partner organisms. Especially the distribution patterns of *Cand.* Paceibacteria appeared to be independent of specific partners, as they primarily showed interconnections among themselves and to other *Cand.* Patescibacteria. The contrasting preferences for hydrochemical conditions among several groups of these organisms prevented general conclusions about parameters driving their distribution. The fermentative metabolism postulated for some members of the *Cand.* Paceibacteria (Brown et al., 2015; Nelson and Stegen, 2015) would provide independence of inorganic electron acceptors, and could explain their ubiquitous predominance throughout the groundwater flow system. The availability of resources not targeted in this study, such as essential organic monomers many *Cand.* Paceibacteria are not able to synthesize themselves due to the lack of metabolic pathways (Brown et al., 2015), might be a stronger driver of their distribution. The high abundance of transporter and glycoside hydrolase genes described (Brown et al., 2015; Castelle et al., 2017; Danczak et al., 2017), together with the high surface-to-volume ratio of ultra-small cells, can be seen as optimizations for the uptake of the low concentrations of such compounds in the oligotrophic groundwater.

Previous studies suggested that most *Cand.* Patescibacteria are anaerobes based on the lack of respiratory chains (Castelle et al., 2018). However, we found surprisingly high abundances of *Cand.* Patescibacteria in oxic groundwater. Moreover, the distribution patterns of the abundant *Cand.* Kaiserbacteraceae, *Cand.* Giovannonibacteraceae, and *Cand.* Nomurabacteraceae across the two aquifer assemblages pointed to contrasting preferences for oxic or anoxic conditions, suggesting differences in the potential utilization of electron acceptors besides a fermentative life style, or in the ability to deal with oxidative stress (Léon-Zayas et al., 2017). Similarly, the presence of nitrite reductase encoding genes in genomes of *Cand.* Patescibacteria has been ascribed to nitrite detoxification mechanisms rather than anaerobic respiration or denitrification (Castelle et al., 2018).

In contrast to *Cand.* Paceibacteria, candidate class ABY1 and *Cand.* Gracilibacteria shared a higher number of positive correlations with taxonomic groups other than *Cand.* Patescibacteria in our network analysis, suggesting that these groups shared ecological niches with other bacterial groups or showed a higher level of dependency on the positively correlated taxa. Interestingly, both classes were also more enriched in the 0.2 μm fraction compared to the majority of order- or family- level taxonomic groups within the *Cand.* Paceibacteria. This might indicate larger cell sizes as previously suggested for *Cand.* Gracilibacteria ("*Cand.* Peregrinibacteria"; Castelle et al., 2018), or could hint to a proclivity for aggregation, including associations with other microorganisms. For *Cand.* Paceibacteria, *Cand.* Gracilibacteria, and candidate class ABY1, network analyses revealed a significant co-occurrence with autotrophic organisms involved in nitrogen, sulfur, and iron cycling. Unfortunately, interpretations on parasitic or symbiotic interactions with these autotrophic taxa remain currently highly speculative: To date, symbiotic interactions have only been experimentally demonstrated for one member of *Cand.* Paceibacteria, *Cand.* Sonnebornia yantaiensis (Gong et al., 2014), which is part of a three-member consortium including an autotrophic partner (*Chlorella*). Given the high relative abundance of some autotrophic groups in the groundwater of the Hainich CZE (Herrmann et al., 2015; Kumar et al., 2017), potential direct interactions would have strong implications for subsurface carbon cycling, as part of the carbon fixed by autotrophy would be shuffled through the abundant *Cand.* Patescibacteria biomass.

CONCLUSION

In conclusion, we propose key mechanisms leading to the success of *Cand.* Patescibacteria in groundwaters. Our novel findings demonstrate: (1) specific order- and family- level groups within *Cand.* Patescibacteria are preferentially mobilized from soils into the groundwater, (2) where they ultimately increase in relative abundance and become the dominant microbial groups, and finally (3) we suggest that spatial differentiation of *Cand.* Patescibacteria in the groundwaters of the Hainich CZE is driven by hydrochemical parameters, resource availability supporting a fermentative lifestyle, as well as interactions with and potential dependence on other bacterial taxa, including abundant autotrophic groups.

AUTHOR CONTRIBUTIONS

MH and KK designed this study. KT and KK designed the Hainich Critical Zone Exploratory. KT, RL, and KL

established field infrastructure and provided groundwater and seepage samples, and provided hydrochemical data and data of Nanoparticle Tracking Analysis. MH performed most of the molecular work and sequence analysis. CEW carried out co-occurrence network analysis. PG performed the bacterial starvation experiments. MT performed statistical analyses. LY performed the calculation of community assembly mechanisms. MT, MH, and KK wrote the manuscript with contributions from all other authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01407/full#supplementary-material

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4. Genome-inferred spatio-temporal resolution of an uncultivated Roizmanbacterium reveals its ecological preferences in oligotrophic groundwater

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Uncultivated microorganisms, including putatively symbiotic bacteria of the Candidate Phyla Radiation (CPR), often dominate microbial communities in various environments. Increasingly available genomic information provided insight into their potential lifestyle, but their exact role in the environment remains enigmatic. We combined the analysis of a draft genome of a member of the CPR, *Cand.* Roizmanbacterium ADI133 (Microgenomates) with the analysis of its spatio-temporal distribution patterns in a complex groundwater ecosystem, using a specifically designed quantitative PCR approach. Genomic characterization and co-occurrence analysis pointed to a host-dependent lifestyle in which *Cand*. Roizmanbacterium provides lactate, generated by fermentation from complex carbon sources, to its host. We hypothesize that *Cand.* Roizmanbacterium ADI133 plays a central role in aquifer biogeochemical cycling by mediating the transfer of organic carbon to and thus stimulating the growth of other community members with important functions in sulphur and nitrogen cycling. Our findings demonstrate the importance of analyzing genomic data in the context of the ecosystem and natural microbial community they were collected from to shed light on the role of yet uncultivated microorganisms in their environment.

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Genome-inferred spatio-temporal resolution of an uncultivated Roizmanbacterium reveals its ecological preferences in groundwater

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Summary

Subsurface ecosystems like groundwater harbour diverse microbial communities, including small-sized, putatively symbiotic organisms of the Candidate Phyla Radiation, yet little is known about their ecological preferences and potential microbial partners. Here, we investigated a member of the superphylum Microgenomates (*Cand*. Roizmanbacterium ADI133) from oligotrophic groundwater using mini-metagenomics and monitored its spatio-temporal distribution using 16S rRNA gene analyses. A Roizmanbacteria-specific quantitative PCR assay allowed us to track its abundance over the course of 1 year within eight groundwater wells along a 5.4 km hillslope transect, where Roizmanbacteria reached maximum relative abundances of 2.3%. In-depth genomic analyses suggested that *Cand*. Roizmanbacterium ADI133 is a lactic acid fermenter, potentially able to utilize a range of complex carbon substrates, including cellulose. We hypothesize that it attaches to host cells using a trimeric autotransporter adhesin and inhibits their cell wall biosynthesis using a toxin–antitoxin system. Network analyses based on correlating *Cand*. Roizmanbacterium ADI133 abundances with amplicon sequencing-derived microbial community profiles suggested one potential host organism, classified as a member of the class *Thermodesulfovibrionia* (Nitrospirae). By providing lactate as an electron donor *Cand*. Roizmanbacterium ADI133 potentially mediates the transfer of carbon to other microorganisms and thereby is an important connector in the microbial community.

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Introduction

Subsurface environments, including aquifers, harbour more than half of the microbial biomass on Earth (Flemming and Wuertz, 2019). Of the estimated 5×10^{27} prokaryotic cells globally inhabiting groundwater (McMahon and Parnell, 2014; Flemming and Wuertz, 2019) a considerable fraction of up to 54% can pass through 0.2 μm pore size filters (Miyoshi *et al*., 2005; Luef *et al*., 2015; Herrmann *et al*., 2019). The majority of these small-sized bacteria are members of the Candidate Phyla Radiation (CPR), a major branch in the tree of life that almost completely lacks cultivated representatives (Wrighton *et al*., 2012; Brown *et al*., 2015; Hug *et al*., 2016), with just a few representatives isolates of species-level lineages within the phylum Saccharibacteria (Cross *et al*., 2019), it is estimated that the CPR comprises up to 26% of the total bacterial diversity on our planet (Parks *et al*., 2017; Schulz *et al*., 2017) with more than 70 different phylum-level lineages. Many of these taxa have been elusive since insertions in the 16S rRNA genes of many CPR (Brown *et al*., 2015) or mismatches with commonly used primers impede detection by 16S rRNA gene amplicon sequencing (Eloe-Fadrosh *et al*., 2016; Schulz *et al*., 2017).

The ubiquitous nature of CPR organisms only became apparent with the increased use of genome-resolved metagenomics (Wrighton *et al*., 2012; Brown *et al*., 2015; Hug *et al*., 2016). The metabolic limitation of CPR organisms, including the common lack of genes for amino acid, nucleotide and lipid biosynthesis due to genome-streamlining, suggests a symbiotic or at least partner dependent lifestyle (Brown *et al*., 2015; Luef *et al*., 2015; Castelle and Banfield, 2018; Probst *et al*., 2018a). CPR bacteria comprise a large

fraction of the groundwater in a karstic aquifer system of the Hainich Critical Zone Exploratory (CZE) located in central Germany (Herrmann *et al.*, 2015; Kumar *et al.*, 2017) and their spatial distribution might be driven by interactions with autotrophic bacteria (Herrmann *et al.*, 2019). Similarly, cultivation-based studies pointed to a symbiotic relationship of *Cand.* Parcubacteria and *Cand.* Saccharibacteria with other pro- and eukaryotic microorganisms (Gong *et al.*, 2014; He *et al.*, 2015; Cross *et al.*, 2019).

Although genome-centric metagenomics can bypass some of the limitations of 16S rRNA gene amplicon sequencing-based approaches for community profiling, metagenomics is still only rarely used to track key organisms across space and time and simultaneously monitor single species abundances in the context of the total communities and environmental variation (Hug *et al.*, 2015; Probst *et al.*, 2018b).

In this study, we elucidate the ecophysiology of CPR bacteria in the Hainich CZE within two superimposed aquifer assemblages along a single hillslope transect (Küsel *et al.*, 2016) where long-term monitoring of the groundwater allowed a detailed characterization of hydrochemical zones (Kohlhepp *et al.*, 2017) as well as the reconstruction of microbial community dynamics (Herrmann *et al.*, 2019; Yan *et al.*, 2019). Using a cell sorting-based approach targeting small cells and coupling it to mini-metagenomic sequencing (McLean *et al.*, 2013), we were ultimately able to reconstruct the genome of an abundant representative of the CPR in oligotrophic groundwater. We combined the characterization of the genome of a *Cand.* Roizmanbacterium with a specific quantitative PCR approach to track its spatio-temporal distribution in oligotrophic groundwater over 1 year. Our findings suggest that *Cand. Roizmanbacterium ADI133* is a potentially symbiotic member of the microbial community that might interact with *Thermodesulfovibrionia* (Nitrospirae), which is a key player in nitrogen as well as sulphur cycling at our study site.

Table 1. Overview of the primers used for amplicon sequencing as well as quantitative PCR.

Primer	Sequence (5′-3′)	References
926Fw	AAA CTY AAA KGA ATT GRC GG	An *et al.* (2013)
1392R	ACG GGC GGT GTG TRC	An *et al.* (2013)
341F	CCT ACG GGN GGC WGC AG	Herlemann *et al.* (2011)
785R	GAC TAC HVG GGT ATC TAA TCC	Herlemann *et al.* (2011)
Bac8Fmod	AGA GTT TGA TYM TGG CTC AG	Loy *et al.* (2002)
Bac338Rabc	GCW GCC WCC CGT AGG WGT	Daims *et al.* (1999)
Roiz341F	CCT ACG GGA GGC AGC AAT CA	This study
Roiz785R	GAC TAC GCA GGT CTC TAA TCT	This study

Results and discussion

Previous investigations of two aquifer assemblages in the Hainich CZE have shown high abundances and diversity of members of the CPR across a transect of 10 groundwater wells at five locations (Herrmann *et al.*, 2019). Here, we used fluorescence-activated cell sorting (FACS) to collect 500 small-sized cells from groundwater of one representative well (Fig. S1) for subsequent reconstruction of genomes of abundant CPR organisms using mini-metagenomic sequencing approach (McLean *et al.*, 2013).

Cell sorting and mini-metagenomic sequencing

A screening of the sorted cell population by amplicon sequencing of 16S rRNA genes with two different primer sets (Table 1) confirmed that the majority of cells belonged to yet uncultivated bacterial phyla (Fig. S2). Notably, 12% of these sequences originating from presumably small-sized cells were affiliated with *Cand.* Roizmanbacteria, belonging to the superphylum Microgenomates. *Cand.* Roizmanbacteria have previously not been detected in long-term monitoring of groundwater from this study site using 16S rRNA gene-targeted amplicon sequencing (Küsel *et al.*, 2016; Kumar *et al.*, 2017; Herrmann *et al.*, 2019). Although the groundwater community is dominated by members of the CPR, Microgenomates made up only 0.7% of the bacterial community (Herrmann *et al.*, 2019).

Through the applied mini-metagenomic approach, we were able to reconstruct the draft genome of a *Cand.* Roizmanbacterium that in the following is referred to as *Candidatus* Roizmanbacterium ADI133 (Fig. S3, Table S1). The genome has a length of 1.2 Mbp is estimated to be 89% complete and does not include any redundant single-copy genes (Brown *et al.*, 2015). The *Cand.* Roizmanbacterium ADI133 genome consists of 85 contigs, has an average GC content of 33.8% and a protein-coding density of 91.2% (Table S2). Equally, low GC contents can be observed in other Roizmanbacteria genomes (Table S3) and point towards an adaptation to nutrient-poor environments where smaller genomes with low GC content are decreasing the costs of DNA replication (Mann and Chen, 2010).

Abundance of Cand. Roizmanbacterium ADI133 across the Hainich CZE

The 16S rRNA gene of *Cand.* Roizmanbacterium ADI133 identified in the reconstructed genome facilitated the design of a specific quantitative PCR assay (Table 1, Fig. S4) that allowed the quantification of *Cand.* Roizmanbacteria. An *in silico* test of the newly designed primer set showed that it

matches the 16S rRNA gene sequence of other *Cand*. Roizmanbacteria as well as other groups within the Microgenomates. However, amplicon sequencing using this primer set revealed a high specificity to *Cand*. Roizmanbacterium ADI133 within the microbial communities of our study site (Fig. S4), allowing us to specifically quantify this particular organism.

To track its abundance across the two aquifer assemblages, water samples from eight wells at four different sites were sequentially filtered through 0.2 and 0.1 μm filters. Even though members of the superphylum Microgenomates have previously been described to be more likely retrieved from the 0.1 μm filter fraction (Castelle *et al*., 2018), over the course of 1 year *Cand*. Roizmanbacterium ADI133 was exclusively detected on 0.2 μm pore size filters except for one sampling event of well H52, where *Cand*. Roizmanbacterium ADI133 comprised 0.18% of the bacteria that passed through the 0.2 μm filter (Fig. S5). Although the retention of *Cand*. Roizmanbacterium ADI133 on the 0.2 μm filters could point towards a larger cell size of this CPR bacterium, the small genome size, as well as the targeted sorting of small cells for the mini-metagenomics approach, suggest a CPR-typical cell size of less than 0.2 μm. The almost complete absence of *Cand*. Roizmanbacterium ADI133 from the 0.1 μm pore size filter fraction indicates a stable attachment to other cells or particles in the groundwater. Cell–cell attachments of CPR cells with other bacteria have previously been demonstrated by electron microscopy (Luef *et al*., 2015) and postulated by various studies analysing potential symbiotic relationships of CPR bacteria (Gong *et al*., 2014; Soro *et al*., 2014; He *et al*., 2015), implying that also *Cand*. Roizmanbacterium ADI133 lives as an episymbiont within the aquifer system.

Over the course of 1 year, the abundance of *Cand*. Roizmanbacterium ADI133 spanned almost three orders of magnitude difference across the transect, ranging from 5×10^4 gene copies L^{-1} at well H43 to 1×10^7 gene copies L^{-1} at well H53 (Fig. 1A). However, the abundance of *Cand*. Roizmanbacterium ADI133 within each well was stable (Fig. S5). Being present in 94% of all samples across the entire transect with a relative abundance of at least 0.1%, *Cand*. Roizmanbacterium ADI133 constitutes a member of the groundwater core microbiome. With respect to the total abundances of bacterial 16S rRNA genes in the groundwater (including Roizmanbacterial 16S rRNA genes, Fig. S5) *Cand*. Roizmanbacterium ADI133 accounted for up to 2.3% of the bacterial community (maximum abundance in well H41, June 2017, Fig. 1B), which is in stark contrast to previous findings of other groundwater ecosystems, where the most abundant member reached less than 1% of the entire community (Castelle *et al*., 2013). Estimated absolute abundances of Roizmanbacteria were highest at wells H52 and H53, under anoxic conditions. Here, the large distance to recharge areas along with long groundwater travel times and the depletion of organic electron donors and oxygen (Kohlhepp *et al*., 2017) may have resulted in the establishment and maintenance of a specific, well-adapted groundwater bacterial community dominated by a few key players, among which the Roizmanbacteria appear to play an important role.

Carbohydrate metabolism

Cand. Roizmanbacterium ADI133 possesses an almost complete genetic machinery for glycolysis as wells as gluconeogenesis, however, the two genes converting glucose to fructose-6-phosphate (Fig. 2A) are absent in this genome. On the one hand, this could be a result of the genome not being complete, but on the other hand, the absence of these genes has previously been described for many members of the CPR (Castelle *et al*., 2018). Unlike most CPR, the genome of *Cand*. Roizmanbacterium ADI133 encodes a phosphofructokinase. Although a pyruvate-kinase is missing in its genome, *Cand*. Roizmanbacterium ADI133 can subsequently use pyruvate to ferment it to lactate, as previously described for members of the CPR (Wrighton *et al*., 2014). The produced lactate is likely excreted and thus could support the growth of, e.g., sulphate-reducing bacteria (Oyekola *et al*., 2009), making *Cand*. Roizmanbacterium ADI133 an important member of the microbial community. In fact, sulphate-reducing bacteria have been found to be abundant in groundwater of the wells H52 and H53 (Schwab *et al*., 2017; Wegner *et al*., 2018), coinciding with the highest abundances of *Cand*. Roizmanbacterium ADI133.

In addition, the genome of *Cand*. Roizmanbacterium ADI133 encodes a cytochrome c as well as the cytochrome c oxidase (subunit II) and an ATP synthase potentially serving as a fragmentary respiratory chain (Fig. 2A). Under oxic or microoxic conditions, electrons could be transported to the cytochrome c using a cupredoxin protein and be further transported to the cytochrome c oxidase that generates H_2O by using oxygen as the final electron acceptor, generating a proton gradient that fuels ATP synthesis. The presence of a simple respiratory machinery makes *Cand*. Roizmanbacterium ADI133 metabolically more versatile and can explain the observed high abundance of this organism in the oxic groundwater wells.

Furthermore, the genome of *Cand*. Roizmanbacterium ADI133 encodes 85 carbohydrate-active enzymes (CAZymes) (Table S4) including glycoside hydrolases (GH), glycosyltransferases (GT), carbohydrate-binding modules (CBM), as well as a carbohydrate esterase (CE). Five of the CAZymes were also identified as putatively secreted proteins (Table S5), potentially involved in the degradation of plant and algal detritus (GH5) as well as bacterial necromass (GT39) (Orsi *et al*., 2018).

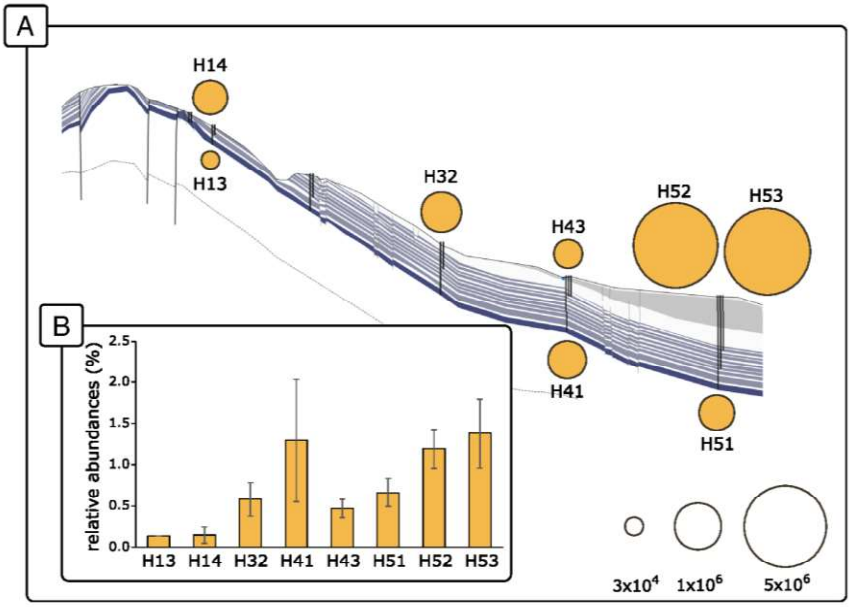

Fig. 1. A. Absolute abundances of *Cand*. Roizmanbacterium ADI133 in the 0.2 µm fraction of each groundwater well across the transect based on a quantitative PCR averaged over sampling campaigns in July 2016, October 2016, January 2016, April 2017 and June 2017. B. Relative abundances of *Cand*. Roizmanbacterium ADI133 compared to the total bacterial community of the 0.2 µm fraction. The cross-section is modified from Kohlhepp *et al*. (2017).

Moreover, multiple genes encode CBM50 modules that are described to be attached to enzymes targeting peptidoglycan. Interestingly, the majority of the detected CAZymes are GTs belonging to the families 2, 4 and 83 associated with glycolipid synthesis. *Cand*. Roizmanbacterium ADI133, like other CPR (Kantor *et al*., 2013), is not able to synthesize glycerolipids but could substitute phosphate groups in externally derived lipids with sugars to build up its own membrane.

Notably, we discovered the presence of genes involved in cellulose degradation. The *Cand*. Roizmanbacterium ADI133 genome encodes a cohesin-dockerin module, the basis of cellulosomes. Cellulosomes mediate the attachment to insoluble plant material and the subsequent degradation of cellulose. On the same scaffold, the genome of *Cand*. Roizmanbacterium ADI133 encodes a cellulase to hydrolyse glycosidic bonds in cellulose. Though the ability to breakdown cellulose has been hypothesized for various members of the CPR (Wrighton *et al*., 2014; Castelle *et al*., 2018) the potential presence of cellulosomes has only been described for members of the *Saccharibacteria* and *Parcubacteria* (Kantor *et al*., 2013; Starr *et al*., 2018). Another extracellular protein potentially involved in the breakdown of plant-derived polysaccharides by *Cand*. Roizmanbacterium ADI133 is an amylopullanase. Amylopullanases have previously been identified to be involved in the breakdown of various saccharides including starch, glycogen and pullulan in a lactic acid-fermenting bacterium (Vishnu *et al*., 2006) but have not been described to be present in CPR. While the potential to break down plant-derived polymers has previously been hypothesized for members of the CPR (Wrighton *et al*., 2014), Taubert *et al*. (2019) were able to demonstrate the degradation of (hemi)cellulose as well as starch by CPR bacteria using a combination of enzyme activity quantification and DNA stable isotope probing in groundwater samples from the Hainich CZE. However, studies analysing the isotopic composition of microbial phospholipid fatty acids at this site indicated that the majority of the microbial community is utilizing old organic carbon (OC) derived from the aquifer rock to build up biomass, despite the presence of younger OC (Schwab *et al*., 2019), especially at the wells with highest abundances of Roizmanbacteria. Hypothetically, the enzymatic machinery of *Cand*. Roizmanbacterium ADI133 to break down plant polymers could also be involved in the degradation of old OC compounds, followed by the release of lactate as a final degradation product. Consequently, by transforming old OC into labile OC easily accessible to other members of the groundwater microbial community, *Cand*. Roizmanbacteria would play a central role in the aquifer carbon flow. However, the proposed mechanism currently remains highly speculative.

The presence of genes encoding CAZymes indicates that besides plant debris or potentially old OC, bacterial necromass might serve as an alternative carbon source for the Roizmanbacterium. However, biopolymers such as those derived from necromass preferentially adsorb to mineral particles and thus, their bioavailability rapidly decreases after cell lysis (Miltner *et al*., 2012; Orsi *et al*., 2018). Alternatively, the same genetic machinery that

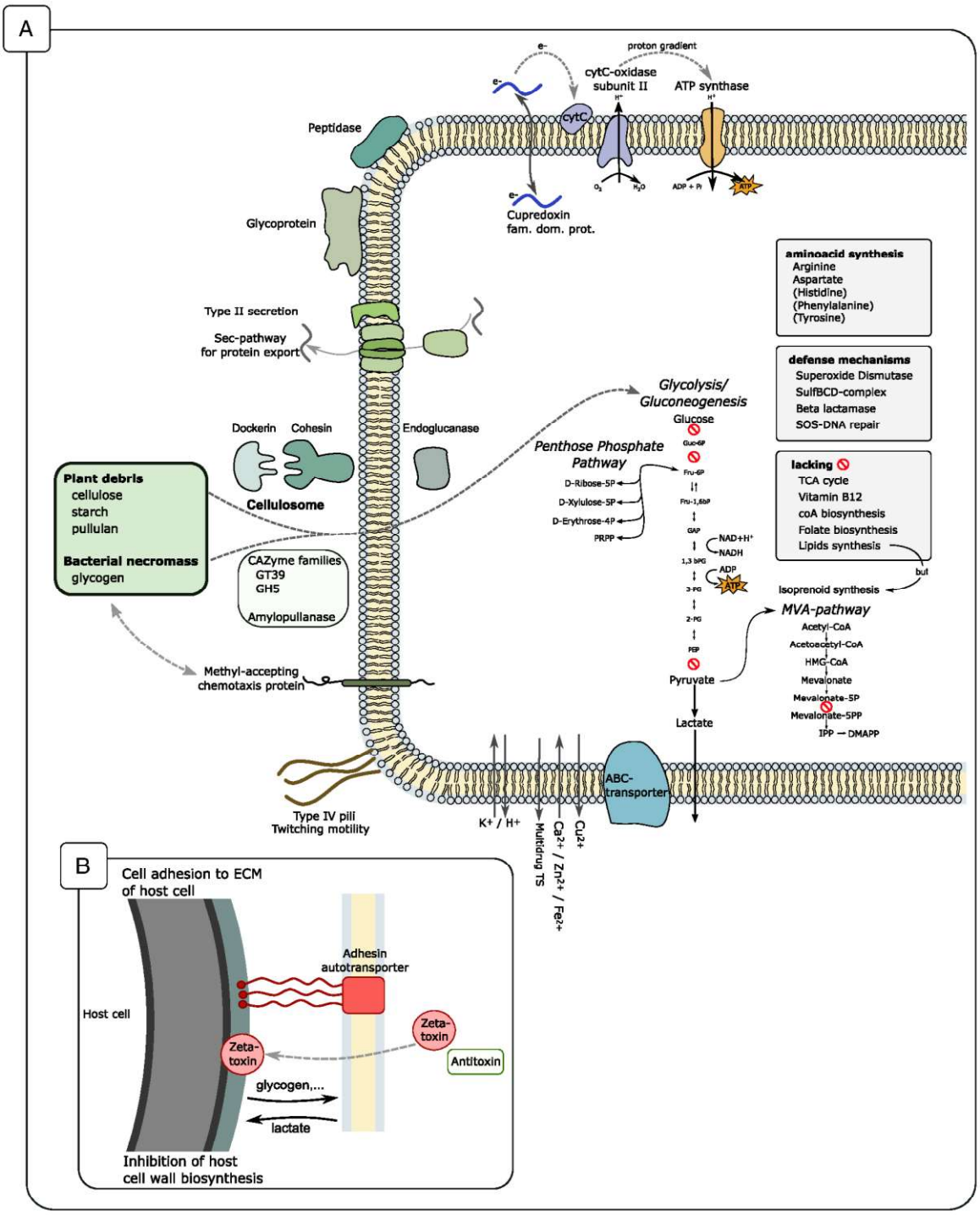

Fig. 2. A. Overview of the biosynthetic capabilities of *Cand.* Roizmanbacterium ADI133. Cytochrome C (cytC); Glucose-6-phosphate (Guc-6P); Fructose-6-phosphate (Fru-6P); Fructose-1,6-biphosphate (Fru-1,6-bP); glycerinaldehyde-3-phosphate (GAP); 1,3-biphosphoglycerate (1,3 bPG); 3-phosphoglycerate (3-PG); 2-phsophoglycerate (2-PG); phosphoenolpyruvate (PEP); carbohydrate-activated enzymes (CAZymes); Isoprenoids isopentenyl pyrophosphate (IPP); Dimethylallyl pyrophosphate (DMAPP). Red signs indicate missing genes from the respective pathways. B. After adhering to the host cell using an adhesion autotransporter system, the zeta toxin produced by *Cand.* Roizmanbacterium ADI133 could inhibit cell-wall biosynthesis of the host. *Cand.* Roizmanbacterium ADI133 can subsequently utilize cell wall components like glycogen from its host in a potential exchange for lactate.

would enable *Cand*. Roizmanbacterium ADI133 to degrade necromass could also be involved in obtaining sugars and lipids from cell walls and membranes of live host cells in a close physical relationship. This direct interaction would be beneficial in a potential symbiotic relationship where a direct uptake of cell components from a host could take place.

Virulence and host association

The genome of *Cand*. Roizmanbacterium ADI133 encodes multiple proteins that are related to cell–cell interactions, such as glycoproteins as well as Type IV pili and fimbria. Both pili and fimbria can be used to attach to surfaces as well as to potential host cells. In addition, *Cand*. Roizmanbacterium ADI133 possesses a gene potentially encoding an autotransporter adhesin that has previously been shown to contain all features of a trimeric autotransporter adhesin (TAA) (Bentancor *et al*., 2012). TAAs are important virulence factors in pathogenic Gram-negative bacteria and play a crucial role in the colonization of, typically eukaryotic, host cells (Linke *et al*., 2006).

In addition, the genome contains type II toxin/antitoxin (TA) system-related genes, respective antitoxins and a zeta toxin that can inhibit cell wall biosynthesis (Mutschler *et al*., 2011). TA systems are usually located on transferable genetic elements like plasmids where they guarantee that only daughter cells that inherit the plasmid survive (Mochizuki *et al*., 2006). CPR bacteria have not been described to possess plasmids (Castelle and Banfield, 2018), suggesting an alternative reason for maintaining a TA system in their otherwise streamlined genomes. After attaching to the host, the zeta toxin could affect the hosts' cell wall biosynthesis and thereby cause a release of required substances like amino acids, lipids or vitamins that the Roizmanbacterium is not able to synthesize itself (Fig. 2B). Alternatively, the encoded TA systems in *Cand*. Roizmanbacterium ADI133 could be a protective mechanism against phages that have recently been discovered in Roizmanbacterial genomes (Chen *et al*., 2019). As soon as the antitoxin is no longer produced during phage infection, the more stable toxin still present in the cell would induce the death of the cell and thereby the proliferation of phages would be prevented (Pecota and Wood, 1996; Emond *et al*., 1998; Fineran *et al*., 2009).

By comparing 128 publicly available Roizmanbacteria genomes (Table S3) to the genome of *Cand*. Roizmanbacterium ADI133, we found that 17 of these genomes encode similar TA system, zeta toxin and at least one antitoxin (Fig. S3). Other than zeta toxins and genes encoding the TA system, whose presence is restricted to closely

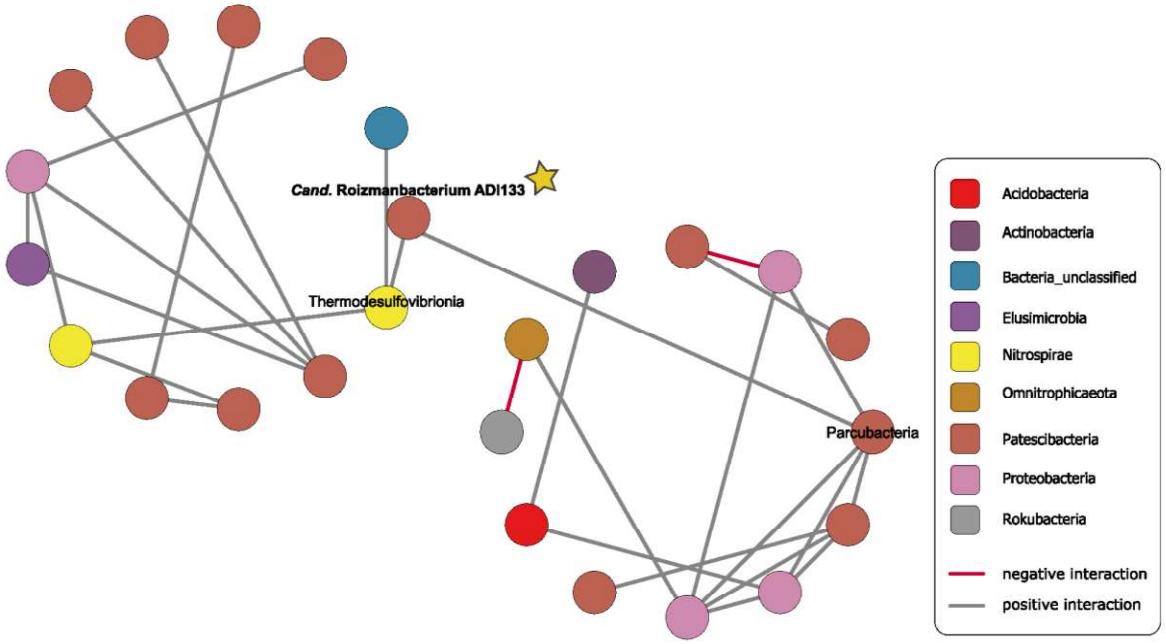

Fig. 3. Correlations of *Cand*. Roizmanbacterium ADI133 (indicated by a star symbol) with other OTU from the analysed groundwater samples. Co-occurrence analysis was carried out with the 300 most abundant bacterial OTUs across eight wells and five sampling time points. Absolute abundances of each OTU as well as *Cand*. Roizmanbacterium ADI133 were calculated based on qPCR and amplicon sequencing of 16S rRNA genes. Here, only network clusters that have interactions with *Cand*. Roizmanbacterium ADI133 are shown. The full network, including all OTUs, can be found in Fig. S6. The network was plotted using Cytoscape 3.7.1.

Table 2. Closest relatives of OTU000150, an uncultivated member of class Thermodesulfovibrionia (Nitrospirae) based on a BLASTn search of the representative 16S rRNA gene sequence for this OTU.

	Identity (%)	Accession	References
Cand. Magnetobacterium bavaricum	88.59	FP929063.1	Jogler *et al*. (2010)
Cand. Magnetoovum mohavensis	87.56	GU979422.1	Lefèvre *et al*. (2011)
Thermodesulfovibrio yellowstonii DSM 11347	86.60	CP001147.1	Bhatnagar *et al*. (2015)

related genomes, antitoxins are conserved across the entire phylum suggesting that strategies to interact with putative host cells or to ward off phage infections are a common feature across Roizmanbacteria.

Co-occurrence of Cand. *Roizmanbacterium ADI133 with other bacteria*

Attachment of *Cand*. Roizmanbacterium ADI133 cells to a specific partner or host bacterium could be reflected in an explicit spatio-temporal co-occurrence pattern along the two aquifer assemblages. Therefore, we determined correlations between the absolute abundances of *Cand*. Roizmanbacterium ADI133 determined via qPCR and the most abundant 300 bacterial Operational Taxonomic Units (OTU) previously identified via 16S rRNA gene amplicon sequencing (Yan *et al*., 2019). Across a total of 29 samples, co-occurrence between *Cand*. Roizmanbacterium ADI133 and two other OTUs was identified (Fig. 3). In addition to a yet uncultivated Thermodesulfovibrionia (OTU 000150) belonging to the Nitrospirae phylum, we detected a positive correlation to another member of the CPR (OTU 000215, *Cand*. Parcubacteria). Previous co-occurrence analyses at the same study site have shown that members of the CPR are often positively correlated to each other, most likely pointing towards similar ecological preferences rather than a physical interaction (Herrmann *et al*., 2019). Although co-occurrence network analyses can give first hints on potential partner-organisms, the relevance of unspecific interactions with other members of a complex community should also be considered.

The Thermodesulfovibrionia-OTU identified as a potential host was distantly related to *Cand*. Magnetobacterium bavaricum [88.6% 16S rRNA gene sequence identity, Table 2 (Jogler *et al*., 2010)], a sulphur-oxidizing magnetotactic bacterium (Vali *et al*., 1987; Petersen *et al*., 1989; Jogler *et al*., 2010). Other members of the class Thermodesulfovibrionia have been shown to harbour diverse metabolic capacities, including the ability to use Dissimilatory Nitrate Reduction to Ammonium in anaerobic respiration (Jogler *et al*., 2010; Lefèvre *et al*., 2011; Arshad *et al*., 2017). Anaerobic metabolisms involving redox reactions of nitrogen and sulphur compounds have recently been identified as dominant metabolic pathways (Kumar *et al*., 2017; Wegner *et al*., 2018) at groundwater wells with the highest abundances of *Cand*. Roizmanbacterium ADI133 and Thermodesulfovibrionia, H52 and H53. However, the potential Nitrospirae host was not observed in all the samples included in the co-occurrence analysis, suggesting that *Cand*. Roizmanbacterium ADI133 might also be associated with other partner organisms but without significant co-occurrence patterns. By utilizing lactate provided by *Cand*. Roizmanbacterium ADI133 as an electron donor (Arshad *et al*., 2017) the *Thermodesulfovibrionia*-bacterium would benefit from an interaction with the CPR bacterium, potentially resulting in a stimulation of its growth. Given the fact that Thermodesulfovibrionia are likely involved in sulphur and nitrogen cycling (Arshad *et al*., 2017, Zecchin *et al*. 2018), *Cand*. Roizmanbacterium ADI133 could indirectly impact biogeochemical cycling within this complex environment.

Conclusions

We combined the analysis of the draft genome of a member of the CPR, *Cand*. *Roizmanbacterium ADI133*, with a quantitative PCR approach that enabled us to follow spatial and temporal distribution patterns of this organism across a complex groundwater ecosystem. The CPR-typical streamlined genome of *Cand*. Roizmanbacterium ADI133, as well as multiple host associations and virulence factors, pointed to a symbiotic lifestyle of this organism, as previously suggested for members of the CPR. Moreover, *Cand*. Roizmanbacterium ADI133 is probably able to utilize OC derived from plant or necromass to generate energy during fermentation to lactate. By providing lactate as a carbon source to potential hosts, it could mediate the transfer of carbon to other community members, including potential key players in important aquifer biogeochemical cycles, such as sulphur and nitrogen cycling. Thereby, *Cand*. Roizmanbacterium ADI133 is not only an abundant member of the microbial community but potentially also acts as an important link in the carbon flow of this subsurface environment.

Experimental procedures

Study site and sampling

Here, we studied pristine groundwater of two superimposed limestone aquifer assemblages, located in the Hainich region in northwest Thuringia where a groundwater monitoring transect, following a downhill slope, was established within the CRC AquaDiva (Küsel *et al*.,

2016). Several groundwater wells access the two main aquifers at five sites along the transect. Groundwater was sampled over a period of 1 year from June 2016 to July 2017 using a submersible motor pump (MP1, Grundfos, Denmark).

Sample preparation for cell sorting and screening of cell pools

Groundwater samples for later cell sorting were obtained in April 2017. Two litres of groundwater from well H53 were filtered through sterile 0.1 μm pore size PES filters. Filters were then transferred to sterile 50 ml tubes containing 40 ml unfiltered groundwater from the same site and were horizontally shaken at 15°C overnight to detach cells from the filter. The resulting cell suspension was conserved by cryofixation using Glycerol-TE-buffer at a final concentration of 10% (Rinke *et al*., 2014), frozen on dry ice, and stored at −80°C. Cryo-fixated samples were stained with SYBR Green following the protocol given in Rinke *et al*. (2014), treated with mild sonication for 5 s and were subjected to cell sorting into 384 well plates (single cells, 50, 100 or 500 cells per well) using a BD FACS Aria III. We focused on cells originating from presumably small cell size populations as indicated by the FSC and SSC versus FITC scatter plots (Fig. S1: populations P1 and P2). Sorted cells were then subjected to whole genome amplification using REPLI-g single cell kit (Qiagen, Hilden, Germany) with added SYTO to follow DNA amplification in real-time on a CFX384 Real-time PCR system (Biorad) (Kaster *et al*., 2014). Reactions were set up in 384 well plates using an EDC Biosystems liquid handler.

Prior to metagenomic sequencing, the composition of the cell pool was determined through high-throughput sequencing of 16S rRNA genes using the primer combinations 341F/785R as well as 926Fw/1392R [(Herlemann *et al*., 2011; An *et al*., 2013; Cole *et al*., 2013), Table 1]. The generation of barcoded amplicons and amplicon sequencing using the Illumina MiSeq platform and V3 Chemistry (Illumina) was performed as previously described (Kumar *et al*., 2018). Sequence analysis of bacterial 16S rRNA amplicons was performed using Mothur (v.1.39.1) (Schloss *et al*., 2009), following the Mothur MiSeq SOP (Kozich *et al*., 2013) along with the SILVA bacteria reference alignment v132.

Sequencing, assembly, binning and annotation of mini-metagenomes

Aiming for a final insert size of ~350 bp DNA was sheared using a Bioruptor® Plus Sonicator (Diagenode, Belgium) at high intensity for 20 cycles (30 s/30 s). Libraries were prepared using the NEBNext® Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Germany) and sequenced on an Illumina HiSeq 2000 in paired-end mode (2 × 150 bp) by GATC Biotech (Konstanz, Germany).

All reads were quality screened and trimmed using BBduk (https://sourceforge.net/projects/bbmap/) and assembled using SPAdes 3.12.0 (Bankevich *et al*., 2012) with the single-cell flag intended for data that has previously undergone multiple displacement amplification. Vizbin (Laczny *et al*., 2015) was used for the binning of the genome, considering all scaffolds that were at least 1000 bp long. Completeness and redundancy of the genome were estimated within Anvio (Eren *et al*., 2015) using the CPR-specific single copy marker gene set proposed by Brown *et al*. (2015). The bin was manually refined using the GC content of assigned scaffolds and subsequently annotated by the MicroScope annotation platform (Médigue *et al*., 2017).

Analysis of 128 publicly available Cand. *Roizmanbacteria genomes*

In total 128 available *Cand*. Roizmanbacteria genomes were downloaded from the database of the National Centre for Biotechnology Information. A Phylogenetic tree, including the *Cand*. Roizmanbacterium ADI133 was calculated based on 32 ribosomal proteins (Fig. S3) using FastTree (Price *et al*., 2009), implemented within Anvio (Eren *et al*., 2015), and visualized using iTOL (Letunic and Bork, 2016).

In order to identify the occurrence of TA systems within *Cand*. Roizmanbacteria, we screened all 128 Roizmanbacteria genomes for the presence or absence of the TA system-related proteins found in *Cand*. Roizmanbacterium ADI133 (Table S7). A cut-off of 60% sequence similarity was set.

Sample preparation for microbial community profiling

Groundwater from all wells was sequentially filtered on a 0.2 μm and a 0.1 μm polycarbonate filters (Nuclepore, Whatman) to collect microbial biomass. All filters were immediately stored on dry ice until they were transported to the laboratory and frozen at −80°C until DNA was extracted using the MoBio Power Soil DNA isolation kit (MO BIO Laboratories, CA, USA) following the manufacturer's protocol. DNA was stored at −20°C until further downstream analyses. DNA samples have been analysed by Herrmann *et al*. (2019) and Yan *et al*. (unpublished) (Table S6).

Quantitative PCR

To follow the abundances of *Cand*. Roizmanbacterium ADI133 across space and time, the primers 341F and 785R (Herlemann *et al*., 2011), which have multiple

mismatches against the 16S rRNA gene sequences of *Cand*. Roizmanbacterium ADI133, were modified so that they unambiguously matched the corresponding sequence of the binding sites in the 16S rRNA gene of *Cand*. Roizmanbacterium ADI133 (Roiz341F and Roiz785R, Table 1, Fig. S4). A quantitative PCR assay was established using the following cycling conditions: 10 min denaturation at 95°C, followed by 45 cycles of 30 s at 95°C, 1 min at 60°C and 25 s at 72°C. The specificity of the primer set was verified by amplicon sequencing of the resulting PCR product at different annealing temperatures (Fig. S5). Standard curves were generated using a plasmid containing a cloned *Cand*. Roizmanbacteria 16S rRNA gene obtained from groundwater of the same aquifer system.

Furthermore, the abundances of total bacterial SSU rRNA genes were determined using the primer combination Bac8Fmod/Bac338Rabc (Daims *et al*., 1999; Loy *et al*., 2002) using cycling conditions previously described (Herrmann *et al*., 2012). All quantitative PCR analyses were performed on an Mx3000P instrument (Agilent, Böblingen, Germany) using Brilliant II SYBR® Green QPCR Master Mix (Agilent).

Co-occurrence networks

By combining information about absolute abundances of all bacteria as well as *Cand*. Roizmanbacterium ADI133 based on quantitative PCR results with relative abundances of the OTUs present in each sample based on amplicon sequencing, we estimated the absolute abundances of each OTU, as well as *Cand*. Roizmanbacterium ADI133 within each sample.

A phylogenetic molecular ecological network (pMEN) was calculated using MENA (Deng *et al*., 2012) based on the Pearson Correlation Coefficient and without log transformation. A cut-off of 0.56 was set by MENA using a random matrix theory-based approach to automatically define the pMEN. Only OTUs that were present in at least 14 of the 29 samples were used. Only modules that have interactions with *Cand*. Roizmanbacterium ADI133 were shown. The network was plotted using Cytoscape 3.7.1 (https://cytoscape.org/). The full network, including all OTUs, can be found in Fig. S6.

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Data Availability

Sequence data were deposited at the European Nucleotide Archive (https://www.ebi.ac.uk/ena/) under BioProject number PRJEB33610. Previously published data can be accessed through the respective BioProject numbers (Table S6).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supporting Information.

Supplemental Figure 1. Flow cytometric analysis of groundwater sample H53 after staining with SYBR Green I, SSC-H vs FITC-A (top) and FCS-H vs FITC-H scatter plots. Particle populations distinguished for subsequent cell sorting are shown in different colours and depicted as P1 (red), P2 (green), P3 (blue). (H53: only P1 to P3).

Supplemental Figure 2. Taxonomic composition of the cell pool used for mini-metagenomic sequencing based on 16S rRNA sequence reads generated with two different primer sets targeting different regions of the 16S rRNA gene (Table 1).

Supplemental Figure 3. Phylogenetic tree based on 32 ribosomal proteins showing the relation of the bin recovered in this study compared to 128 Roizmanbacteria genomes available in public databases. *Cand*. Roizmanbacterium ADI133 is highlighted in orange. Genomes encoding a T/A system as well as the respective toxin and antitoxins are indicated by green boxes. For collapsed nodes the number of included leaves is given. The tree was calculated using FastTree (Price *et al*., 2009) implemented within Anvio (Eren *et al*., 2015) and visualized using iTOL (Letunic and Bork, 2016).

Supplemental Figure 4. (A) Overview of the different primer sets used in this study. Highlighted in circles are the number of mismatches each primer has against the 16S rRNA gene of *Escherichia coli* (grey) and *Cand*. Roizmanbacterium ADI133 (orange). The primer set Roiz341F/Roiz785R is a modified version of the primers 341F/785R (Herlemann *et al*., 2011). (B) Primer specificity of Roizmanbacteria specific primer set at different annealing temperatures was verified by amplicon sequencing of the respective PCR products using the MiSeq (Illumina) platform. Sequence analysis of bacterial 16S rRNA amplicons was performed using Mothur (v.1.39.1) (Schloss *et al*., 2009), following the Mothur MiSeq SOP (Kozich *et al*., 2013) along with the SILVA bacteria reference alignment v132.

Supplemental Figure 5. Absolute abundances of 16S rRNA genes per Litre for the total bacterial community as well as *Cand*. Roizmanbacteria (A) averaged over n samplings in the 0.2 μm and 0.1 μm filter fraction and (B) for each sampling campaign separately in the 0.2 μm filter fraction.

Supplemental Figure 6. Correlations of *Cand*. Roizmanbacterium ADI133 (indicated by star symbol) with other Operational Taxonomic Units (OTU) from the analysed groundwater samples. The 300 most abundant OTUs were picked for the five time points at eight wells. Absolute abundances of each OTU as well as *Cand*. Roizmanbacterium ADI133 were calculated based on qPCR. The network was calculated using MENA (Deng *et al*., 2009) using Pearson Correlation Coefficient, no log transformation and a cut-off of 0.56. Only OTUs that were presents in 14 of the 29 samples were used. The network was plotted using Cytoscape 3.7.1.

Table S1. Average Nucleotide Identity (ANI) of the eight most closely related publicly available Roizmanbacteria genomes with Cand. Roizmanbacterium ADI133. ANI values were calculated using FastANI (Jain *et al*., 2018).

Table S2. Characteristics of the draft genome of Cand. Roizmanbacterium ADI133.

Table S3. Overview of the 128 publicly available Roizmanbacteria genomes used in this study.

Table S4. Overview of all CAZymes identified within MicroScope using the dbCan database.

Table S5. Overview of genes encoding putatively secreted proteins identified within MicroScope using the SignalP.

Table S6. Overview of the sequence data used to construct co-occurrence networks. All sequence data has been deposited in the European Nucleotide Archive (ENA).

Table S7. List of gene accessions for genes/proteins/enzymes highlighted in Fig. 2.

5. Bacterial necromass is rapidly metabolized by heterotrophic bacteria and supports multiple trophic levels of the groundwater microbiome

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Manuscript draft in preparation

Microbial communities in subsurface environments are facing limited inputs of fresh organic carbon. Heterotrophic microorganisms must adapt to such oligotrophic conditions developing mechanisms that allow a fast and efficient uptake of sparse nutrients, for example the reduction of cell size, or lowering general metabolic costs by genome streamlining. Recently, the relevance of the recycling of dead microbial biomass (necromass) for parts of the microbial community has been demonstrated in different environments. However, the potential of groundwater heterotrophs to metabolize necromass and the consequences for the entire groundwater microbiome, lacks fundamental understanding. In this study we investigated the effects of necromass addition to the microbial community in fractured bedrock groundwater. Using a combined DNA and Protein Stable Isotope Probing approach we followed the uptake of ^{13}C-labeled necromass from a groundwater isolate by the bacterial and eukaryotic groundwater community. Heterotrophic members of the genera *Flavobacterium*, *Massilia*, *Rheinheimera*, *Rhodoferax* and *Undibacterium* rapidly respond to the availability of necromass, presumably by metabolizing necromass-derived amino acids. The release of nitrogen and sulfur compounds during necromass degradation subsequently led to a stimulation of autotrophic community members. Furthermore, groundwater eukaryotes were found to incorporated necromass-derived carbon. Our results suggest that in the groundwater environment, necromass might be a key factor sustaining not only heterotrophic microorganisms, but a large part of the entire groundwater microbiome over multiple trophic levels.

Supplementary data to this article can be found at the end of the manuscript draft.

Bacterial necromass is rapidly metabolized by heterotrophic bacteria and supports multiple trophic levels of the groundwater microbiome

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Microbial communities in subsurface environments are facing limited inputs of fresh organic carbon. Heterotrophic microorganisms must adapt to such oligotrophic conditions developing mechanisms that allow a fast and efficient uptake of sparse nutrients, for example the reduction of cell size, or lowering general metabolic costs by genome streamlining. Recently, the relevance of the recycling of dead microbial biomass (necromass) for parts of the microbial community has been demonstrated in different environments. However, the potential of groundwater heterotrophs to metabolize necromass and the consequences for the entire groundwater microbiome, lacks fundamental understanding. In this study we investigated the effects of necromass addition to the microbial community in fractured bedrock groundwater. Using a combined DNA and Protein Stable Isotope Probing approach we followed the uptake of ^{13}C-labeled necromass from a groundwater isolate by the bacterial and eukaryotic groundwater community. Heterotrophic members of the genera *Flavobacterium*, *Massilia*, *Rheinheimera*, *Rhodoferax* and *Undibacterium* rapidly respond to the availability of necromass, presumably by metabolizing necromass-derived amino acids. The release of nitrogen and sulfur compounds during necromass degradation subsequently led to a stimulation of autotrophic community members. Furthermore, groundwater eukaryotes were found to incorporated necromass-derived carbon. Our results suggest that in the groundwater environment, necromass might be a key factor sustaining not only heterotrophic microorganisms, but a large part of the entire groundwater microbiome over multiple trophic levels.

Keywords: Stable Isotope Probing, Proteomics, Subsurface, AquaDiva

Introduction

Life in the subsurface is shaped by the lack of photosynthesis-driven primary production, leading to a limited availability of organic carbon (OC) in subsurface environments like groundwater (Akob and Küsel, 2011). Microorganisms that inhabit groundwater strongly depend on inputs of fresh OC from the surface, or OC generated by chemolithoautotrophic primary production in the subsurface. However, inputs of surface-derived OC to the subsurface are limited and chemolithoautotrophy often occurs at certain hotspots within the aquifer system (Kumar *et al.*, 2017; Wegner *et al.*, 2019; Herrmann *et al.*, 2020). Thus, microorganisms inhabiting the terrestrial subsurface need to be well adapted to the limited availability of OC.

Microorganisms in the groundwater cope with the oligotrophic conditions by maximizing their capability for the uptake of nutrients (Sowell *et al.*, 2009). Often this is accompanied by a reduction of the genome to lower reproductive and metabolic costs. Consequently, essential metabolic capabilities can be lost. An extreme example for genome streamlining are members of the Candidate Phyla Radiation (CPR), that are frequently found to inhabit groundwater environments (Luef *et al.*, 2015; Proctor *et al.*, 2018; Herrmann *et al.*, 2019). These organisms lack the ability to synthesize amino acids, nucleotides, vitamins or lipids and are proposed to depend on host- or partner organisms to receive components that are essential to build up their cells (Castelle and Banfield, 2018; Castelle *et al.*, 2018). Dead microbial biomass (necromass) can be an easily accessible source of OC for all heterotrophic members of the groundwater microbiome, including the CPR (Orsi *et al.*, 2018; Starr *et al.*, 2018; Geesink *et al.*, 2019).

Consisting mainly of proteins (50 %), RNA (20 %), and small molecules (13%), necromass provides amino acids but also other central metabolites of bacteria, that can easily be taken up and utilized by microorganisms. In contrast to necromass, plant-derived litter that can enter the aquifer system mainly consists of complex polymers. The degradation and metabolization of plant litter requires specific enzyme machineries and thus, has only found to be performed by a small subset of the microorganisms in groundwater (Taubert, Stähly, *et al.*, 2019). Thus, microbial necromass can potentially be utilized by a broader range of microorganisms and should be preferentially recycled in environments with limited availability of OC.

In surface water bodies the concept of biomass recycling has been well described: In the "microbial loop" heterotrophic bacteria use dissolved organic matter (DOM) as an energy source to build up biomass that is subsequently consumed by proto- and metazoa, and transferred to higher trophic levels of the aquatic food chain (Azam *et al.*, 1983). Bacteria are in turn affected by viral lysis (Fuhrman, 1999), leading to the availability of necromass in the environment. Also in soils, the presence of necromass has been well described as one

fraction of DOM that can be accessed by microorganisms as a source of energy or carbon (Miltner *et al.*, 2012; Gleixner, 2013; Ma *et al.*, 2018). In addition, DOM-microaggregates can be taken up by protozoa that are typically predating on bacteria.

The potential to use necromass as a source of OC has been shown in different environments like marine sediments, soils and compost (Miltner *et al.*, 2012; Bradley *et al.*, 2018; Müller *et al.*, 2018; Hanajima *et al.*, 2019; Liang *et al.*, 2019). While these compounds should be accessible to a wide range of microorganisms, studies from marine and terrestrial environments have shown, that only a few taxa are involved in the degradation of necromass in their respective habitat (Müller *et al.*, 2018; Hanajima *et al.*, 2019). A study investigating the interactions within a sulfate-reducing, naphthalene degrading enrichment from groundwater points towards the importance of bacterial necromass when looking at trophic interactions in groundwater consortia that can, analogous to surface water system, form a "subsurface microbial loop" (Dong *et al.*, 2018). However, the fluxes of necromass derived carbon within the microbial community, as well as the impact of necromass recycling in groundwater on complex communities remain elusive.

Here we investigate fractured bedrock groundwater obtained from the Hainich CZE (Küsel *et al.*, 2016) to study the implications of necromass for complex microbial communities in the terrestrial subsurface. We hypothesize that necromass can be metabolized by a broad range of microorganisms, including members of the CPR. By adding necromass of a bacterium previously isolated from the same environment to groundwater mesocosms we aimed at following the uptake of ^{13}C, on DNA and protein-level by a combined Stable Isotope Probing (SIP) approach (Neufeld *et al.*, 2007; Jehmlich *et al.*, 2010). In order to elucidate potential implications of necromass recycling for the entire groundwater microbiome across trophic levels, we used this approach to factor in both, bacterial and eukaryotic community members. Our results reveal, that necromass might be a key factor supporting heterotrophic bacteria as well as a large part of the microbial community in groundwater across trophic levels.

Material and Methods

Labeling and Generation of Necromass

A bacterial isolate (*Pseudomonas sp.;* Geesink *et al.*, 2018) that has previously been isolated from groundwater of the Hainich CZE (Küsel *et al.*, 2016), was grown on S2P solid medium (Geesink *et al.*, 2018) for one day at room temperature as a pre-culture. Subsequently, the bacterium was transferred into Minimal Medium (0.4 g L^{-1} K_2HPO_4, 1.6 g L^{-1} $MgSO_4 \times 7H_2O$, 0.8 g L^{-1} NH_4Cl, 25 mM HEPES, 5 mL trace element solution "T" (Boden *et al.*, 2008)) containing either 0.9 g L^{-1} ^{12}C- or ^{13}C-labeled glucose (Sigma-Aldrich) as carbon source to generate unlabeled and ^{13}C-labeled bacterial biomass.

Cultures were incubated in sterile 50 mL centrifuge tubes (Greiner bio-one) at room temperature with mild shaking for three days. Subsequently, all cultures were centrifuged at 10,000 *g* for 10 min, the cell pellets were washed in 0.1 % NaCl (*w*/*v*) solution and combined in serum bottles under sterile conditions. Cell numbers in both solutions (labeled and unlabeled) were determined using flow cytometry (CyFlow Cube6, Sysmex) and both cell suspensions were diluted to equal cell concentrations.

In order to generate necromass, the cell suspensions underwent three cycles of autoclaving (121 °C, 20 min) following Dong *et al.* (2018). Afterwards, 100 µL of the generated necromass were spread on sterile S2P-plates and incubated for ten days, in order to guarantee that no viable cells were left in the necromass solutions. The necromass solutions were stored at -20 °C until further usage.

Study Site and Groundwater Sampling

The Hainich Critical Zone Exploratory (CZE) is located in the northwest of Thuringia (Germany) and facilitates access to two superimposed aquifer assemblages via multiple groundwater wells (Küsel *et al.*, 2016). In October 2018, groundwater was pumped from one well (H41) using a submersible motor pump (MP1, Grundfos, Denmark) as previously described (Kohlhepp *et al.*, 2017). Groundwater was filled into sterile 10 L glass bottles, closed with Teflon lids (DWK Life Science) and cooled until further processing.

Incubation Setup, DNA and Protein Extraction

In total, 21 10 L bottles were filled with 9 L groundwater directly after sampling. While three bottles were used as T0, the remaining 18 bottles were supplemented with ^{12}C or ^{13}C labeled necromass equivalent to 2×10^8 cells L^{-1}. The water within the bottles without added necromass was directly filterer on 0.1 µm filters (Supor, PALL Corporation) for biomass collection. All other mesocosms were incubated at 15°C in the dark for the entire course of the experiment. Oxygen concentrations were monitored daily using a FDO 925 sensor (WTW; Table S1). On day 2, 4 and 8 the entire water of three ^{12}C- as well as three ^{13}C-mesocosms was filtered as previously described for biomass collection. All filters were directly stored at -80 °C until further processing. The filtrate was collected in autoclaved glass bottles and one liter per mesocosm was stored for metabolomic analyses.

DNA and proteins were extracted from the filters as previously described with slight modifications (Taubert *et al.*, 2018). No beads were added to the lysis solution. Instead, lysis was performed during a 2h incubation at 60°C. DNA pellets were resuspended in 50 µL nuclease free water and store at -20°C until further processing.

Sequencing and Sequence Analysis

To test whether the necromass still contained amplifiable DNA and to assess the bacterial diversity in each mesocosm as well as the

original groundwater at day 0, high-throughput sequencing of 16S rRNA genes using the primer combination 341F/785R (Herlemann *et al.*, 2011) was performed. Sequencing was carried out using the Illumina MiSeq platform and V3 Chemistry (Illumina). Adapter sequences were removed from the raw sequences using cutadapt (Martin, 2011) and the remaining sequences were analyzed using Mothur v.1.39.1 (Schloss *et al.*, 2009), following the Mothur MiSeq SOP (Kozich *et al.*, 2013) along with the SILVA bacteria reference alignment v132.

Proteomics

The obtained protein extracts underwent an SDS polyacrylamide gel electrophoresis as well as a subsequent in-gel tryptic digestion. The purified and concentrated peptides following Taubert *et al.* (2018). Subsequently, the peptides were resuspended in 0.1 % formic acid (*v/v*) for the following LC-MS/MS analysis on a Q Exactive HF instrument (Thermo Fisher Scientific) equipped with a TriVersa NanoMate source (Advion Ltd.) in LC chip coupling mode. A volume of 5 µL of the peptide lysates were separated via an Ultimate 3000 nanoRSLC-system (Dionex/Thermo Fisher Scientific).

Proteome Discoverer (v1.4.0288, Thermo Scientific) was used to identify the proteins and the acquired MS/MS spectra were searched against a custom database created from Uniprot based on taxonomic information of the community composition within the incubations based on 16S rRNA gene amplicon sequencing using the SequestHT algorithm. Following Taubert and Grob *et al.* (2019), trypsin was picked as cleavage enzyme, allowing a maximum of two missed cleavages. A precursor mass tolerance (in MS) of 10 ppm and a fragment mass tolerance (in MS/MS) of 0.05 Da were applied. Carbamidomethylation of cysteine was considered as fixed and oxidation of methionine was set as dynamic modification. The peptide spectrum matches were validated using Percolator (v2.04) with a false discovery rate (FDR) < 1 % and quality filtered for XCorr ≥ 2.25 (for charge state +2) and ≥ 2.5 (for charge state +3). Identified peptides were grouped using the strict parsimony principle (Nesvizhskii and Aebersold, 2005).

The taxonomic classification of peptides was based on the lowest common ancestor method in UniPept (Mesuere *et al.*, 2018). The identification of ^{13}C-labelled peptides as well as the incorporation of ^{13}C were done by comparing measured and expected isotopologue patterns, chromatographic retention times and fragmentation patterns (Seifert *et al.*, 2012; Taubert *et al.*, 2012).

DNA-SIP

DNA from the final time point was used for DNA stable isotope probing (SIP) following Taubert *et al.* (2019). In brief, 2-3 µg of DNA were added to a mixture of Gradient Buffer (0.1 M Tris, 0.1 M KCl, 1 mM EDTA; pH 8) and 7.2 M CsCl and the final density was adjusted to 1.725 g mL^{-1}. Ultracentrifugation in a Sorvall Discovery 90SE (Hitachi) and an NVT 90 rotor was carried out for 70h at 20°C and 44,100 rpm. Subsequently,

the mixture was separated into 13 fractions, fraction densities were calculated based on refractive indices (AR200 refractometer, Reichert technologies, Buffalo, USA) and DNA was precipitated and washed following Taubert *et al.* (2019). The heavy and light DNA fractions of the ^{13}C incubations, as well as the corresponding factions of the ^{12}C incubations, were selected for 16S rRNA gene amplicon sequencing based on their density (ρ_{heavy}=1.73, ρ_{light}= 1.69) following (Neufeld *et al.*, 2007)

Results and Discussion

Responses in the bacterial community to necromass addition

Changes in the bacterial community in the mesocosm incubations supplemented with bacterial necromass were followed by monitoring abundance and community composition using quantitative PCR and amplicon sequencing of bacterial 16S rRNA genes. The number of bacterial 16S rRNA gene copies, as a proxy for cell abundances, increased rapidly within four days after the addition of necromass, from $1.3 \times 10^8 \pm 4.9 \times 10^7$ to $7.0 \times 10^9 \pm 2.6 \times 10^9$ gene copies per liter of groundwater, indicating bacterial growth within the mesocosms (Figure 1). This increase was significant after four and eight days compared to the original groundwater ($p < 0.01$, Student's *t*-test).

Bacterial growth in the mesocosms was accompanied by a decrease in the overall diversity of the community (Figure 2). Especially from the original groundwater to day 2, Shannon diversity decreased significantly from 5.67 ± 0.09 to 3.82 ± 0.38 and continued to decrease marginally at the later time points (Students t-test, Table S2). Similar to previous studies, the groundwater community was dominated by *Nitrospiraceae* and families belonging to the Candidate Phyla Radiation (CPR) superphylum (candidate division UBA9983, *Cand.* Kaiserbacteria, *Cand.* Wolfebacteria, *Cand.* Magasanikbacteria, *Cand.* Parcubacteria, *Cand.* Garcilibacteria; Herrmann *et al.*, 2019; Yan *et al.*, 2019). After two days of incubation, relative abundances of *Flavobacteriaceae, Burkholderiaceae* and *Pseudomonadaceae* contributed up to 68 % of the bacterial community. *Spirosomaceae* and *Alteromonadaceae* increased in relative abundance at the later time points, reaching 5.7 % at day 8 (Figure 2). While only comprising 2.5 % of the original groundwater community, these families remained dominant in the mesocosms till the end of the incubation. In contrast, *Nitrospiraceae*, which made up 14.2 % of the original groundwater community, decreased to 3.6 % after two days and disappeared at the later time points. Although the addition of necromass led to a drastic change in the community composition, the predominantly responding genera are of relevance within the original groundwater community.

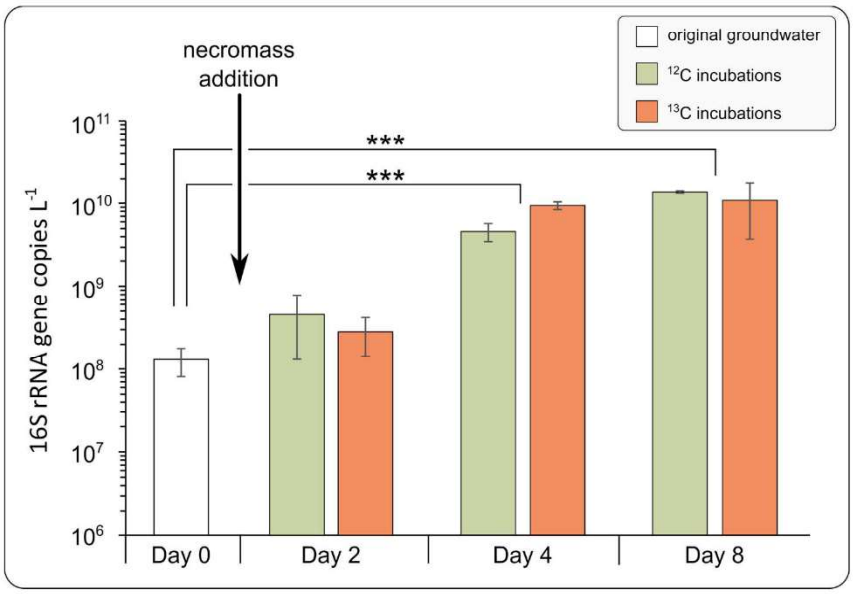

Figure 1 Bacterial abundances in groundwater and mesocosms supplemented with necromass. Average 16S rRNA gene copy numbers within the original groundwater (white), as well as within the incubations at day 2, 4 and 8 after necromass addition in the ^{12}C (green) and ^{13}C (orange) mesocosms. Error bars show standard deviation of three samples. P-values were derived from student's t-test and indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

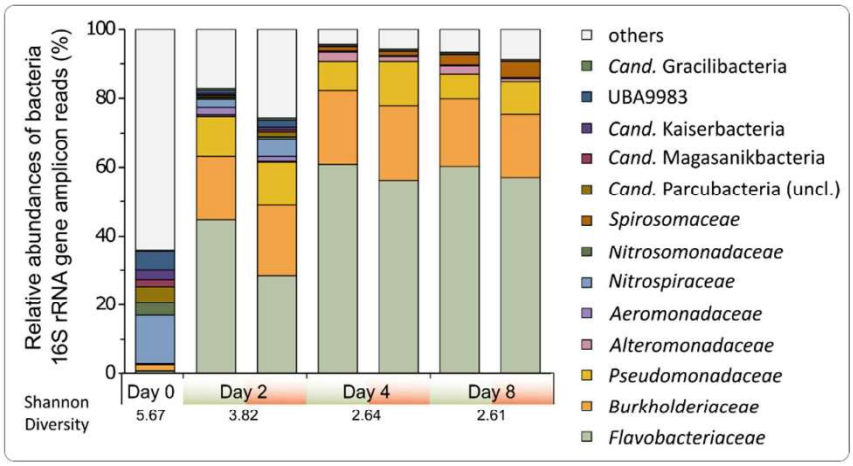

Figure 2 Phylogenetic profiles based on relative abundances of bacterial 16S rRNA gene amplicons. Average relative abundances of bacterial families in the original groundwater, as well as the ^{12}C (green) and ^{13}C (orange) mesocosms over the eight days of incubation. Shannon diversity indices are given as average values per timepoint.

Rapid depletion of Pseudomonas necromass

Among the abundant *Pseudomonadaceae*, multiple OTUs affiliated with the genus *Pseudomonas* were identified. These OTUs might have originated from either the groundwater community, or the supplementation of *Pseudomonas* necromass to the mesocosms. When comparing the enriched OTUs in the mesocosms to the 16S rRNA gene sequence of the *Pseudomonas* isolate Hainich_002 used for generating the necromass, we observed that they were clearly distinguishable (Figure 3A). The most abundant *Pseudomonas* OTUs showed only 95 to 98 % sequence identity to Hainich_002. The OTU with the highest identity (OTU00019; 97.8 %) constituted less than 2 % of the microbial community (Figure 3B). This is in accordance with the observation that no PCR amplicons targeting 16S rRNA genes could be obtained from necromass samples after the triple autoclaving, confirming that the necromass addition did not introduce a bias to our DNA based community profiling, and that no viable cells of Hainich_002 were left in our mesocosms. In contrast, the peptides affiliated with *Pseudomonas* that were detected in the microcosms supplemented with ^{13}C-labeled necromass showed a high ^{13}C relative isotope abundance (RIA) of 98% (average across all peptides at day 2, Figure S1), indicating that they were derived from the necromass.

Compared to *Pseudomonas* OTUs, which only decreased marginally in relative abundance over incubation time, the relative abundance of these peptides decreased rapidly during the incubation, dropping from 56.06 % ± 2.15 % at day 2 to only 2.41 % ± 0.25 % at day 4. This explicit decrease suggested a rapid degradation of necromass by the groundwater microbial community within the first two days of the incubation (Figure 3C).

Uptake of necromass by heterotrophic community members

To follow the uptake of necromass derived carbon in the microbial community, we quantified the ^{13}C relative isotope abundances (RIAs) in peptides of the ten most abundant genera using SIP-metaproteomics. The uptake of ^{13}C-labelled bacterial necromass by heterotrophic members of the groundwater community should have resulted in a high ^{13}C-incorporation in the biomass of these bacteria. In fact, we observed ^{13}C RIAs of 93 to 96% in peptides of the genera *Flavobacterium, Massilia, Rheinheimera, Rhodoferax* and *Undibacterium* already after two days of incubation. These genera are typical heterotrophic organisms in groundwater that are potentially introduced from soil (Herrmann *et al.*, 2019). In the mesocosms, these genera showed a rapid increase in abundance both on 16S rRNA gene level as well as on peptide level. The significant increase in cell numbers observed coincided with the depletion of most peptides, demonstrating the fast response of the community to necromass.

A functional classification of the identified peptides affiliated with the heterotrophic genera

revealed the presence of numerous amino acid transporters (Table S3), pointing towards the use of necromass-derived amino acids as a carbon source by heterotrophic bacteria. Considering that proteins make up a large proportion of necromass, and a rapid depletion of necromass-derived peptides in the incubation was observed to accompany heterotrophic growth, this type of metabolism seems plausible. Furthermore, peptides derived from enzymes of the TCA cycle and gluconeogenesis pathways were identified for the most abundant potentially heterotrophic genera *Flavobacterium*, *Massilia*, *Rheinheimera*, *Rhodoferax* and *Undibacterium* (Table S3). During growth on amino acids, these pathways would be essential to channel carbon into the central metabolism.

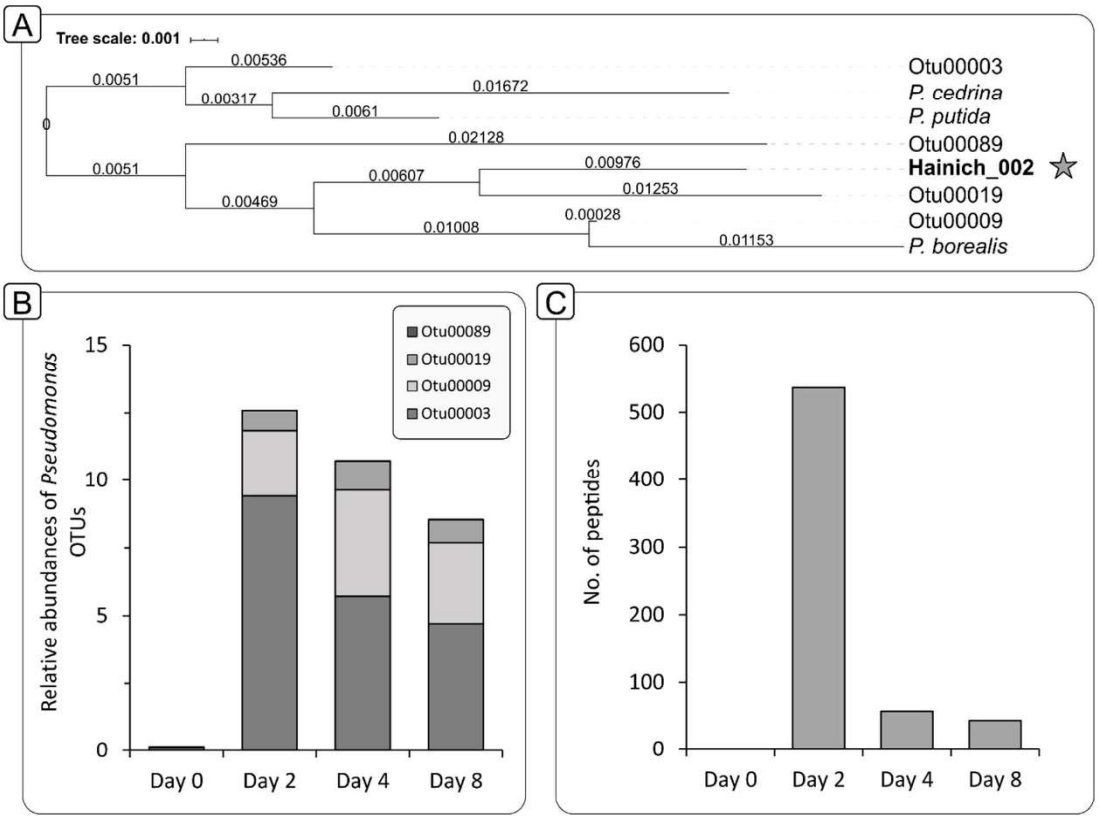

Figure 3 Abundances of *Pseudomonas* on DNA and peptide level. (A) Phylogenetic tree based on 16S rRNA gene sequences of the most abundant *Pseudomonas* OTUs as well the isolate Hainich_002 that was used for necromass generation (star symbol). The tree was calculated using the arb neighbor joining method (1000 bootstraps) within arb (Quast *et al.*, 2012; Yilmaz *et al.*, 2014). Closely related *Pseudomonas* species were added as references. (B) Relative abundances of *Pseudomonas* related OTUs increase after necromass was added on day 0 and then progressively decrease over the course of the incubation. (C) Number of peptides associated with the genus *Pseudomonas* within the original groundwater at day 0, as well as the ^{12}C labeled incubations over time.

Response of putatively autotrophic bacteria

Apart from the clearly heterotrophic genera described above, peptides of several other representatives of the top ten genera did not feature the high ^{13}C RIA expected for growth on necromass. These genera, including *Nitrosomonas, Acidovorax, Limnohabitans*, and *Paucibacte*r, only had between 0.5 and 23 % ^{13}C incorporated in their peptides (Figure 4). The clear growth of these genera, evident from an increase in abundance both on DNA and peptide level, indicated the primary use of an unlabeled carbon source. The largest pool of unlabeled carbon in the mesocosms was bicarbonate derived from the groundwater, present in concentrations around 400 mg L^{-1} (≙ 84 mg ^{12}C L^{-1}) (Lehmann and Totsche, 2019). Compared to the added 0.5 mg ^{13}C L^{-1} derived from necromass, even the complete degradation of the added ^{13}C necromass would only lead to a maximal ^{13}C RIA of 0.6 % in the bicarbonate pool. Thus, a lower incorporation of ^{13}C within an organism points towards the use of bicarbonate as a carbon source by autotrophic bacteria.

Peptides derived from enzymes of the Calvin-Benson-Bassham (CBB) cycle, such as a Ribulose-bisphosphate carboxylase/oxygenase were identified for *Nitrosomonas* in our samples (Table S3). Likewise, members of the genera *Acidovorax, Limnohabitans* and *Paucibacter* have been described to be capable of CO_2 fixation via the CBB cycle (Zeng *et al.*, 2012; Alfreider and Tartarotti, 2019; Taubert, personal communication), supporting the assumption that these organisms might have grown autotrophically in our mesocosms. Furthermore, peptides belonging to proteins linked to nitrogen and sulfur cycling were detected (Table S3). These proteins included an ammonia monooxygenase and nitrite reductase (*Nitrosomonas*), a nitric oxide reductase (*Acidovorax*) and soxAX cytochromes involved in thiosulfate oxidation (*Limnohabitans*) involved in processes that could provide energy for chemolithoautotrophic growth. The metabolization of amino acids by heterotrophic bacteria potentially resulted in the release of reduced nitrogen and sulfur compounds that were subsequently utilized by *Nitrosomonas*, *Acidovorax* and *Limnohabitans*. In agreement, the putative autotrophs primarily increase between day 4 and 8, after the heterotrophic members had already increased between day 2 and 4 (Figure 4).

The amount of incorporated ^{13}C varied between the different genera (Table S4). *Nitrosomonas* (0.99 ± 0.15 % ^{13}C) and *Acidovorax* (1.99 ± 1.18% ^{13}C) were likely growing autotrophically, exclusively assimilating CO_2. The higher RIA within *Limnohabitans* (13.24 ± 4.92% ^{13}C) and *Paucibacter* (5.32 ± 3.40%^{13}C) suggested an assimilation of small amounts of ^{13}C-labeled necromass in addition to a major part of CO_2, indicating a mixotrophic lifestyle. The nature of assimilated organic carbon compounds, however, remained uncertain.

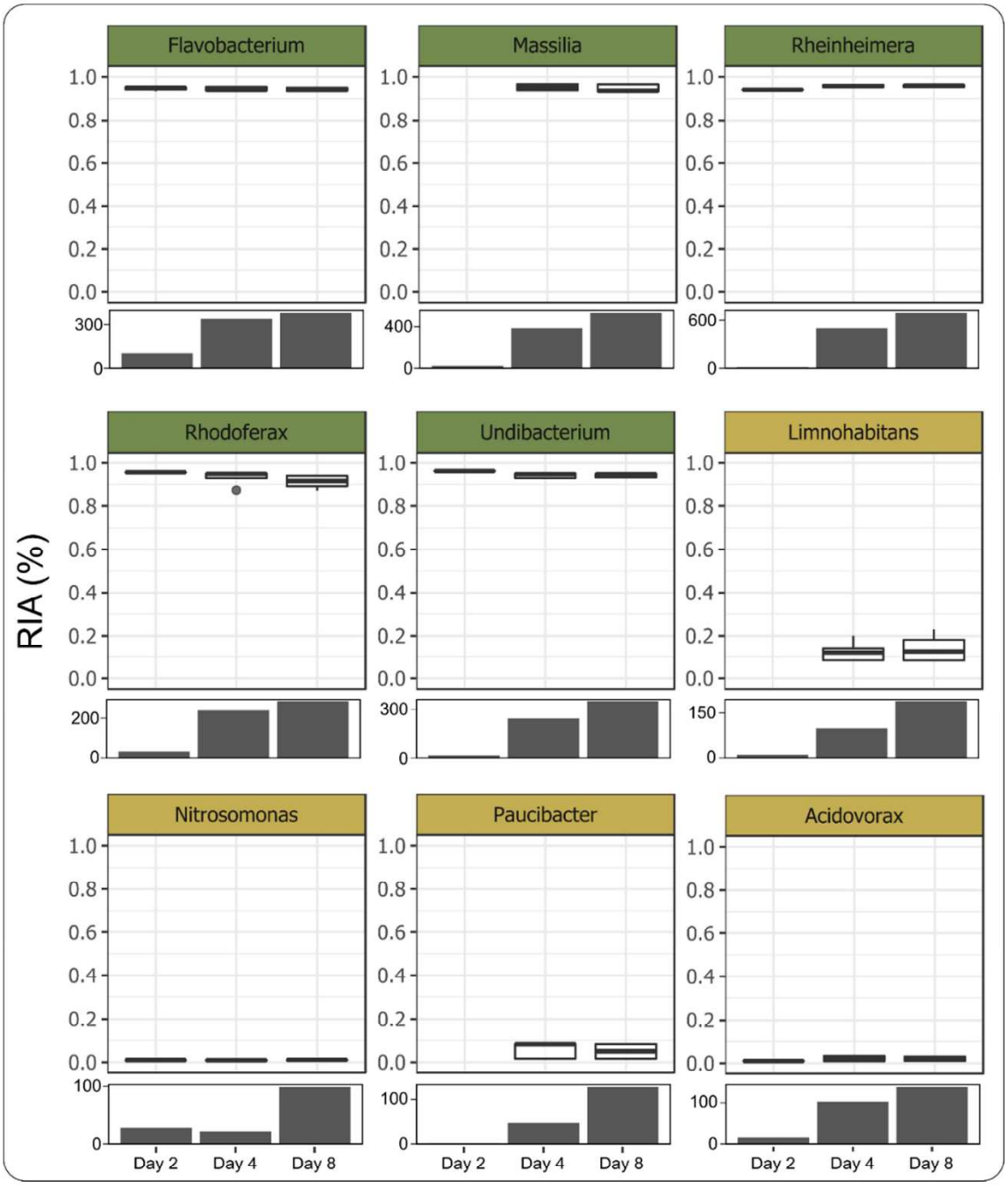

Figure 4 Relative isotopic abundances (RIA) and number of peptides in the most abundant genera. Boxplots show the uptake of labeled carbon by potential heterotrophic (dark green) and autotrophic bacteria (yellow) in the ^{13}C incubations. The corresponding bar charts represent the numbers of identified peptides in the ^{12}C incubations for the respective genus.

Groundwater eukaryotes incorporate necromass-derived carbon

To confirm the results observed and to additionally determine the ^{13}C incorporation in microorganisms not covered by the SIP-metaproteomic approach, we performed DNA-SIP from samples of the mesocosms incubated for eight days. Besides bacterial genera that were identified to take up necromass-derived ^{13}C using Protein-SIP, DNA-SIP revealed a significant enrichment of DNA from operational taxonomic units (OTUs) belonging to the genera *Pseudarcicella, Iodobacter, Chitinimonas, Perlucidibaca* and *Aquabacterium* in the ^{13}C heavy DNA fraction (Figure S2), indicating ^{13}C incorporation. Representatives of these genera are described as heterotrophs and were frequently isolated from aquatic habitats, including the Hainich CZW (Kalmbach *et al.*, 1999; Song *et al.*, 2008; Kampfer *et al.*, 2012; Geesink *et al.*, 2018; Jude, 2019). Furthermore, one OTU belonging to the CPR phylum *Cand.* Gracilibacteria was enriched in the heavy DNA fraction of the ^{13}C incubations (Figure S2). Genomic insights suggest a dependency of *Cand.* Gracilibacteria on the uptake of citrate, malate and amino acids from external sources, due to the lack of glycolysis, the pentose-phosphate and Entner Douderoff pathways (Sieber *et al.*, 2019). These compounds might be provided through the necromass in our mesocosms.

Furthermore, we also observed a strong enrichment of eukaryotic DNA in the ^{13}C heavy DNA fractions, based on qPCR targeting 18S rRNA genes. In the ^{13}C labeled incubations, 84 % of the quantified 18S rRNA gene copies were found in the heavy DNA fraction. The significant difference between heavy and light DNA fractions in the ^{12}C and ^{13}C incubations indicated an incorporation of ^{13}C derived from necromass by the eukaryotic community (Figure 5, Table S5). The increase in biomass of heterotrophic bacteria potentially promoted the growth of bacterivorous protozoa present in the groundwater community, as previously reported (Risse-Buhl *et al.*, 2013). While groundwater eukaryotes either directly fed on the labeled necromass or on the subsequently labeled heterotrophs, our results demonstrated the transfer of necromass-derived carbon through trophic levels and across domains.

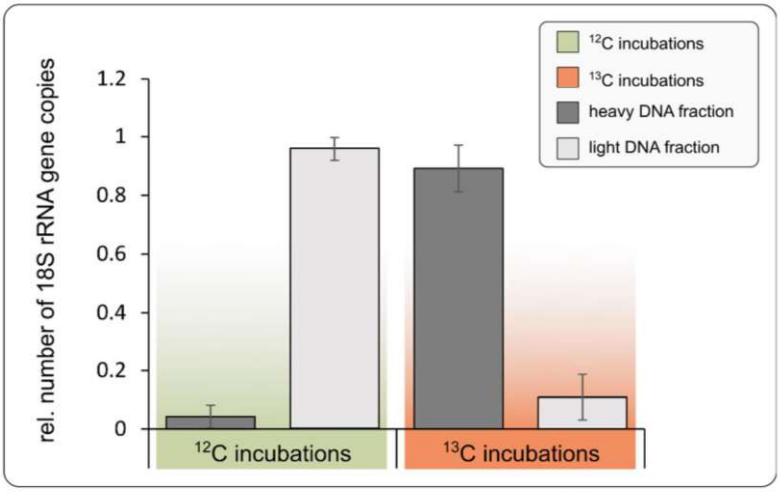

Figure 5 Evidence of necromass uptake by Eukaryotes via DNA-SIP. Relative abundances of eukaryotic 18S rRNA genes in the heavy and light DNA fraction show a significant shift towards the heavy DNA fractions in the incubations with ^{13}C-labeled necromass ($p < 0.001$; Fisher's exact test; Table S5), indicating that eukaryotes have been taking up necromass.

Implications for Groundwater

In our microcosms, heterotrophs directly responded to the increasing availability of necromass by using amino acids as a carbon source. The breakdown of amino acids resulted in release of reduced nitrogen and sulfur compounds that were subsequently utilized by putatively autotrophic community members (Figure 6). A study investigating the release of ammonium by heterotrophic bacteria demonstrated how increasing rates of amino acid turnover increase ammonium excretion and the then increased availability of nitrogen compounds affects the community structure of phototrophic primary producers (Kirchman *et al.*, 1989).

Within the studied groundwater, dissolved organic carbon (DOC) concentrations are low (0.3-5.8 mg L^{-1}, Kohlhepp *et al.*, 2017) and comparable to the amounts of organic carbon provided by necromass within the studied mesocosms (~0.5 mg L^{-1}). Shallow groundwater systems, like the studied Hainich CZE, still receive inputs of matter and microorganisms from the surface (Benk *et al.*, 2019; Herrmann *et al.*, 2019) that are potentially in the range of the added necromass. In fact, seepage water has been shown to contain high amounts of cells that are introduced to the groundwater but likely die under the oligotrophic conditions (Herrmann et al., 2019) and necromass itself can be transported to groundwater from soils (Miltner *et al.*, 2012). Thus, although in lower concentrations, necromass is a plausible carbon source within the groundwater system.

Our results demonstrate, that typical groundwater microorganisms of the Hainich CZE (Herrmann *et al.*, 2019; Yan *et al.*, 2019) are able to rapidly degrade necromass. The subsequent utilization of nitrogen- and sulfur components by autotrophic bacteria within our mesocosms could equally take place in the aquifer system. In fact, the presence and activity of autotrophic bacteria involved in nitrogen and sulfur cycling in groundwater of the Hainich CZE has been demonstrated before and sulfate-reducing bacteria have been proposed to be involved in the turnover of intermediate compounds from the breakdown of organic matter (Kumar *et al.*, 2017; Schwab *et al.*, 2017, 2019; Wegner *et al.*, 2019). These findings suggest that nitrogen and sulfur compounds derived from necromass metabolization could indeed support autotrophic community members in the groundwater of the Hainich CZE.

Furthermore, also eukaryotic microorganisms are incorporating necromass-derived carbon within the studies mesocosms. Groundwater of the Hainich CZE furthermore hosts a large diversity of proto- and metazoa as well as fungi (Risse-Buhl *et al.*, 2013; Nawaz *et al.*, 2018; Herrmann *et al.*, 2020). Lower trophic levels of protozoa are known to feed on bacteria and thus benefit from greater amounts of available bacterial biomass. Within the aquifer system, complex food webs have been found to be coinciding with a higher potential for chemolithoautotrophy (Herrmann *et al.*, 2020). Similarly, a stimulation of hetero- and autotrophic growth by necromass recycling

could positively impact higher trophic levels in the groundwater, supporting the concept of a "subsurface microbial loop" (Dong *et al.*, 2018).

The insights into the recycling of dead microbial biomass by the microbial community in groundwater gained within this mesocosm study demonstrated how necromass derived carbon might impact the entire microbial community in subsurface ecosystems. Our findings suggest that necromass degradation is one potential key process that sustains not only heterotrophic bacteria, but also large parts of the microbial community in groundwater across trophic levels.

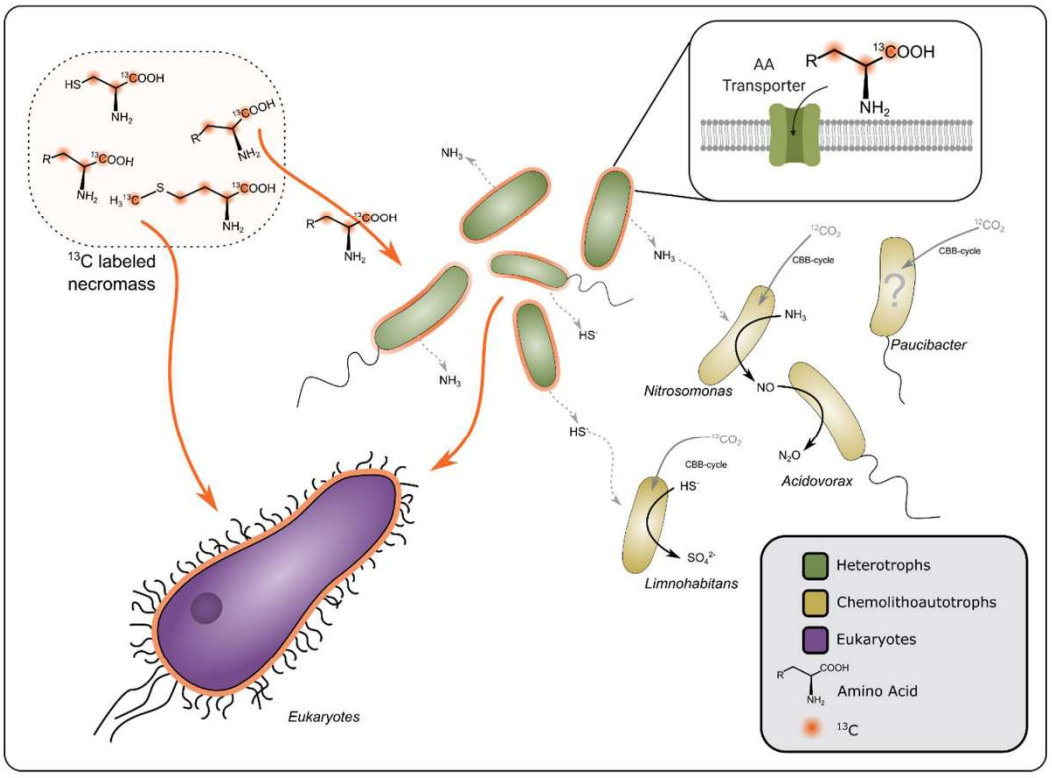

Figure 6 Conceptual view of recycling of necromass derived carbon within the mesocosms. ^{13}C labeled necromass (orange) is taken up by heterotrophic members of the community that can metabolize amino acids. Excess nitrogen and sulfur compounds are being released and can subsequently be metabolized by autotrophic bacteria. Eukaryotes are taking up necromass ^{13}C by feeding on the heterotrophic community members or necromass directly.

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872 ## Supplemental Material

873

874 ### Supplemental Figures

875 **Figure S1** Identified peptides belonging to the genus *Pseudomonas* are mainly derived from necromass.

876

877 **Figure S2** DNA-SIP reveals differences in labeling of bacterial genera on OTU-level.

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880 ### Supplemental Tables

881 **Table S1** Oxygen concentrations within the mesocosms.

882

883 **Table S2** Shannon diversity based on 16S rRNA gene abundances.

884

885 **Table S3** List of identified peptides of interest for the ten most abundant genera.

886

887 **Table S4** Relative isotope abundances (RIA) of ^{13}C within the mesocosms.

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889 **Table S5** Significance values derived for 18S rRNA gene abundances in the heavy and light fractions of the ^{12}C
890 and ^{13}C incubations

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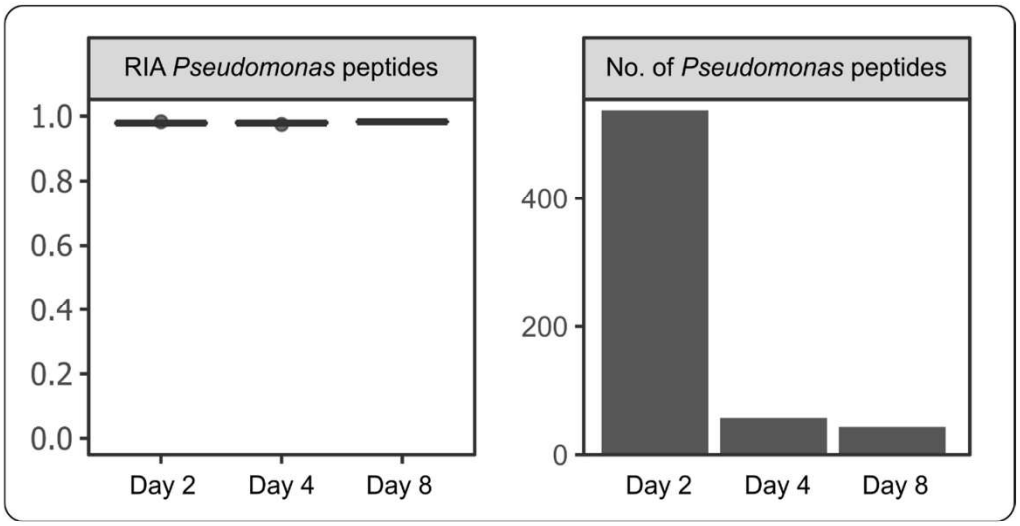

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896 **Figure S1 Identified peptides belonging to the genus *Pseudomonas* are mainly derived from necromass.** (A)
897 Relative isotope abundances (RIA) of ^{13}C in the *Pseudomonas* peptides show consistently show a full labeling
898 throughout the course of the incubation. (B) The number of identified peptides drastically decreases between
899 two and four days of incubation.

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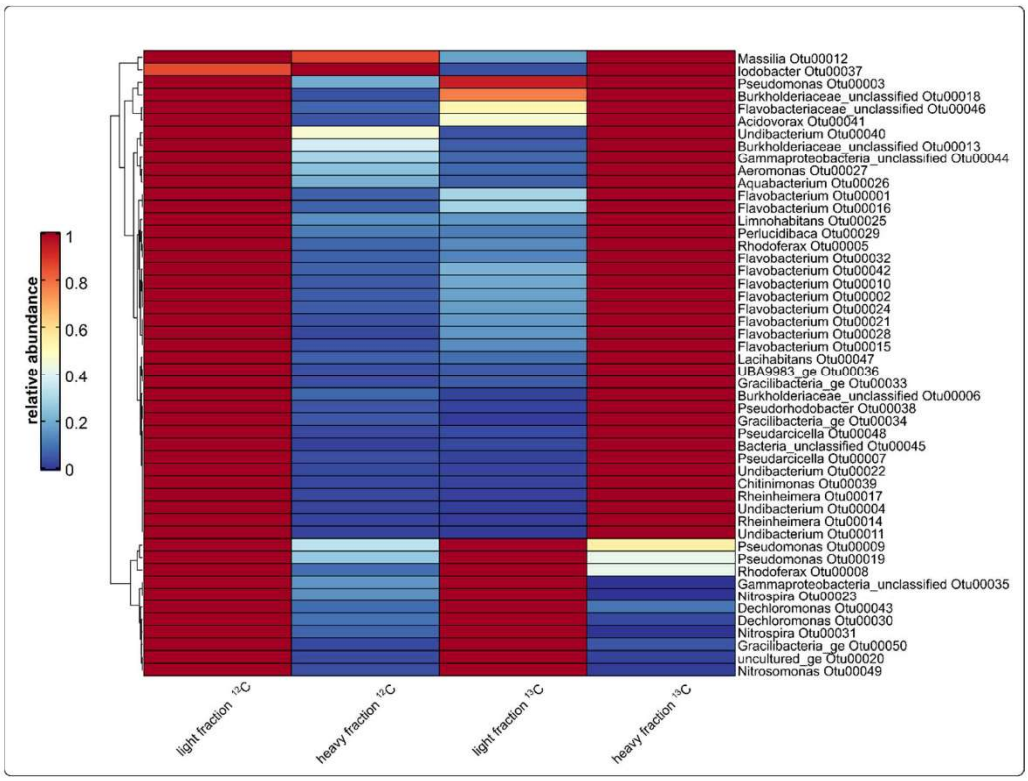

Figure S2 DNA-SIP reveals differences in labeling of bacterial genera on OTU-level. Relative abundance in relation to DNA content of the heavy and light factions in the ^{12}C and ^{13}C incubation for the 50 most abundant OTUs.

Table S1: Oxygen concentrations within the mesocosms were monitored daily over the course of the incubation.

	Oxygen concentration (mg/L)							
Mesocosm	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**
SIP_4	8.90	9.05						
SIP_5	8.75	9						
SIP_6	8.72	8.88						
SIP_7	9.09	9.15						
SIP_8	8.89	9.09						
SIP_9	8.89	9.04						
SIP_10	9.12	9.13	9.25	9.26				
SIP_11	9.04	9.2	9.29	9.27				
SIP_12	9.31	9.35	9.40	9.56				
SIP_13	8.85	9.06	9.10	9.29				
SIP_14	8.96	9.1	9.14	9.28				
SIP_15	9.15	9.26	9.24	9.36				
SIP_16	9.12	9.25	9.25	0.39	9.20	8.88	9.29	9.45
SIP_17	9.13	9.3	9.28	9.40	9.21	8.95	9.34	9.33
SIP_18	9.05	9.15	9.26	9.22	9.14	9.98	9.31	9.34
SIP_19	9.27	9.32	9.28	9.36	9.25	9.04	9.56	9.57
SIP_20	9.38	9.64	9.42	9.55	9.35	9.14	9.55	9.49
SIP_21	9.29	9.41	9.32	9.44	9.25	9.11	9.47	9.43

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Table S2: Shannon Diversity of 16S rRNA genes within the original groundwater (day 0) and the different mesocosms at day 2-8. Significant differences were calculated with respect to the diversity in the original goroundwater.

Mesocosm	nseqs	shannon diversity	Day	Average Diversity	Standard deviation	Significance (T-Test)
SIP_1	7554	5.586192		5.67433	0.09495	
SIP_2	8813	5.806141	Day 0			
SIP_3	11105	5.630669				
SIP_4	7744	3.516432		3.82042	0.38432	0.00004
SIP_5	10594	3.388719				
SIP_6	9410	3.465864	Day 2			
SIP_7	9963	4.169094				
SIP_8	9216	4.397577				
SIP_9	7000	3.984845				
SIP_10	7975	2.625738		2.63937	0.10853	0.000002
SIP_11	7501	2.624142				
SIP_12	7497	2.860058	Day 4			
SIP_13	9082	2.549183				
SIP_14	9149	2.524334				
SIP_15	9397	2.652743				
SIP_16	8715	2.665079		2.60685	0.08461	0.00001
SIP_17	7763	2.629818				
SIP_18	9377	2.715129	Day 8			
SIP_19	10044	2.530193				
SIP_20	7108	2.463644				
SIP_21	9455	2.63724				

913

Table S3: Selection of identified peptides that are further discussed within the manuscript.

Peptide sequence	Accession	Genus	Cbb3-type cytochrome c oxidase subunit
DPQAMAIGER	A0A1I2FPJ7	*Acidovorax*	Malate dehydrogenase
IIGMGGALDSSR	V6SHC1	*Flavobacterium*	Citrate synthase
LFILHADHEQNCSTSTVR	V6SLJ0	*Flavobacterium*	Aconitate hydratase
AEFGINPGSEQIR	V6SRK3	*Flavobacterium*	Monofunctional lysine-ketoglutarate reductase
YELFNLPK	V6SPS4	*Flavobacterium*	Fumarate hydratase class I
NIIEDPEATDNDR	V6STY6	*Flavobacterium*	Phosphoenolpyruvate carboxykinase (ATP)
GVTQPEPTFSACFGK	V6SSR1	*Flavobacterium*	Enolase
AAANELGMPLYR	V6SM28	*Flavobacterium*	Phosphoglycerate kinase
IEAAKPTIDK	V6STT4	*Flavobacterium*	2,3-bisphosphoglycerate-independent phosphoglycerate mutase
VHFLGLVSDGGVHSHTSHLR	V6SS19	*Flavobacterium*	Glyceraldehyde-3-phosphate dehydrogenase
GHLETIHAYTNDQNLVDNMHK	V6SXX7	*Flavobacterium*	Triosephosphate isomerase
EIFSKPDVDGGLIGGAALK	V6SJF8	*Flavobacterium*	SoxAX cytochrome complex subunit A
TASQGIDEYR	A0A0N7J8C1	*Limnohabitans*	Phosphoenolpyruvate carboxylase
LTVADEIDNALSYYR	A0A086W9M6	*Massilia*	Phosphoenolpyruvate synthase
NASLGEMISQLASAGVR	A0A085EQ87	*Massilia*	Enolase
KVQLVGDDLYVTNTK	A0A1M5K3Z9	*Massilia*	Glyceraldehyde-3-phosphate dehydrogenase
EVLTYQTEPLVSIDFNHNPASSNFDSTLTK	A0A0Q6WJC6	*Massilia*	Fructose-1,6-bisphosphatase class 1
YVDEMLAGK	A0A098UB85	*Massilia*	Pyruvate dehydrogenase E1 component
LLEMVANMTDDDIWR	A0A0Q6VPY7	*Massilia*	Pyruvate kinase
AEAIPVLQEILDASDGIMVAR	A0A098U7P4	*Massilia*	Citrate synthase
DAHPMSVLVGTVGALASFYHDSLDINDAK	A0A086WAJ2	*Massilia*	Aconitate hydratase
YVAEGTPTMIFGGEEYGTGSSR	A0A086WAK2	*Massilia*	Malate dehydrogenase
GVMMEVDDCAFPLLAGMTAHSDPMTAFK	A0A2G8T4Y9	*Massilia*	Fumarate hydratase class I
FEGFSGTVTDAVNEGVR	A0A0Q6WYT4	*Massilia*	NADP-dependent isocitrate dehydrogenase (Fragment)
HMGWIEAADLIIASMEK	A0A353HSV3	*Massilia*	Alcohol dehydrogenase (Cytochrome c)
NGFFYVNDAATGK	A0A4R2VPS6	*Massilia*	Ubiquinol-cytochrome c reductase cytochrome c1 subunit
VGGMMTTALR	A0A1M5SPU2	*Massilia*	Ribulose bisphosphate carboxylase large chain
HTYWTPDYTPLDTDLLACFK	F8GEW5	*Nitrosomonas*	Ammonia monooxygenase subunit B
ITMDITNNGDTGVNIGEFTTAGVR	A0A1I3YYT1	*Nitrosomonas*	Ribulose bisphosphate carboxylase large chain
HTYWTPDYTPLDTDLLACFK	F8GEW5	*Nitrosomonas*	Ribulose bisphosphate carboxylase small chain
KFETFSYLPAMSDK	F8GEW4	*Nitrosomonas*	Ribulose-bisphosphate carboxylase
GWNPAIEHTEPEYVMDSYWYMWK	F9ZF98	*Nitrosomonas*	Ribulose-phosphate 3-epimerase
AGADTFVAGSAIYQAGK	A0A2V3WDZ7	*Nitrosomonas*	Malate dehydrogenase
LDLPAGTDLALYDLAPVTPGVAVDLSHIPTAVK	I1DXT7	*Rheinheimera*	Citrate synthase
TVGWISHWDEMLSDK	F7NXQ0	*Rheinheimera*	Aconitate hydratase B
LGQGANVYLASAELAAVASIIGR	I1DSU0	*Rheinheimera*	Isocitrate dehydrogenase [NADP]
HVEQFVEENHLR	F7NXH7	*Rheinheimera*	Succinate dehydrogenase flavoprotein subunit
GATDSDIQAALAR	A0A1B7UKV8	*Rheinheimera*	Succinate--CoA ligase [ADP-forming] subunit alpha
VICQGFTGSQGTFHSTQALEYGTQMVGGVSPGK	A0A1B7UKV3	*Rheinheimera*	Phosphoenolpyruvate carboxykinase (ATP)
KNEPVIWDAIR	F7NTH3	*Rheinheimera*	Phosphoenolpyruvate synthase
TLSEAEAVIGLLAEQGLK	A0A0U4NDX8	*Rheinheimera*	Enolase
SGETEDSFIADLAVGTAAGQIK	F7NTY6	*Rheinheimera*	Phosphoglycerate kinase
SLCEVDLIPEAQR	A0A1B7UJG3	*Rheinheimera*	Ubiquinol-cytochrome c reductase iron-sulfur subunit
DPNSDEEQQPAYAK	A0A0U4W1Y6	*Rheinheimera*	Malate dehydrogenase (Oxaloacetate-decarboxylating)(NADP+)
IAPAVAQAAADSGVALRPILDMDAYR	A0A1H0LVJ2	*Rhodoferax*	Malate synthase G
QAGPQLVVPVLNAR	A0A1P8KE66	*Rhodoferax*	Citrate synthase
QLFTGSVSR	A0A1W9KYH3	*Rhodoferax*	Aconitate hydratase B
IAPIFYNTMEDSGALPIELDVSQMAMGDVIELRPYDGK	Q21XG8	*Rhodoferax*	Isocitrate dehydrogenase [NADP]
FPGTSGIGIKPVSR	A0A1G3HW44	*Rhodoferax*	Succinate--CoA ligase [ADP-forming] subunit alpha
AGESIFGIPIFASVK	A0A1H0MJQ9	*Rhodoferax*	Fumarate hydratase class I
WGEFTGSLDDAINEGVR	Q21VA3	*Rhodoferax*	Enolase
GISTAVGDEGGFAPNVPNHEAAIQMILEAIDK	A0A3E1R9U8	*Rhodoferax*	Ubiquinol-cytochrome c reductase iron-sulfur subunit
RTPEQVAALAGIEGLLADPK	A0A3D5VMS0	*Rhodoferax*	Malate dehydrogenase
IGKPVAAIEK	A0A3S0IRZ7	*Undibacterium*	Malate synthase
ESVGSGATYDR	A0A431T1W5	*Undibacterium*	Citrate synthase
ATLSFSDGSPSLEMPIYK	A0A3S5HM32	*Undibacterium*	Aconitate hydratase B
IAAMEAWLADPQLLK	A0A3Q9BQ18	*Undibacterium*	Isocitrate dehydrogenase [NADP]
HMGWLEAADLLISATEK	A0A318IZB6	*Undibacterium*	Succinate dehydrogenase flavoprotein subunit
GTMQQYCGVFR	A0A318JFC7	*Undibacterium*	Succinate--CoA ligase [ADP-forming] subunit beta
AFMETDCSLAEINPLILTGSGK	A0A318JC59	*Undibacterium*	Fumarate hydratase class I
FEGFSGSIIDAVNEGVR	A0A3S0IL58	*Undibacterium*	Phosphoenolpyruvate carboxykinase [GTP]
TNFAMLIPAIK	A0A318JW40	*Undibacterium*	Phosphoenolpyruvate synthase
IMLNVGNPQLAFDFQSVPNEGVGLAR	A0A431T3B1	*Undibacterium*	Enolase
GLTTAVGDEGGFAPSVENHESAIK	A0A3Q9BT91	*Undibacterium*	Ubiquinol-cytochrome c reductase cytochrome c1 subunit
EWFGAVPPDLSVIAR	A0A318J376	*Undibacterium*	Ubiquinol-cytochrome c reductase cytochrome c1 subunit

Table S4: Relative Isotope Abundances (RIA) of ^{13}C within the mesocosms. 2 to 7 peptides were manually examined for the incorporation of ^{13}C.

Peptide Sequence	Genus	Day 2			Day 4				Day 8			Average RIA (%)	Standard deviation
		SIP_7	SIP_8	SIP_9	SIP_13	SIP_14	SIP_15	SIP_19	SIP_20	SIP_21			
APSWPNDPNSPTGK	***Acidovorax***				0.033	0.04	0.035	0.035	0.031	0.031		1.99	1.18
LQSEALSAETDAR	***Acidovorax***	0.011	0.011	0.01	0.01	0.01	0.01	0.011	0.01	0.011			
SELIDAIAADAGITK	***Flavobacterium***		0.954	0.951	0.933	0.934	0.937	0.936	0.936	0.934		94.36	0.87
LTNGNEVNAYIPGEGHNLQEHSIVLVR	***Flavobacterium***			0.934	0.94	0.939	0.942	0.94	0.94	0.938			
DALTNVMPHVEVR	***Flavobacterium***				0.957	0.954	0.957	0.955	0.955	0.95			
SDAADIELALDAAHAAADAWGK	***Limnohabitans***				0.084	0.085	0.084	0.083	0.084	0.084		13.24	4.92
IYSEAGIPQISPSATNPK	***Limnohabitans***				0.117	0.12	0.124	0.125	0.124	0.123			
LLNAFPFVTK	***Limnohabitans***					0.183	0.201	0.23	0.217	0.182			
IYNDAGIPQISPSATNPK	***Mas[illegible]***				0.968	0.964	0.967	0.968	0.969	0.965		94.79	1.56
FASTISNLQTTSENLSAAR	***Mas[illegible]***							0.936	0.937	0.934			
VNGVVGHLNSGTTVPASK	***Massilia***				0.938	0.936	0.938	0.932	0.933	0.933			
ITHANYDVPGR	***Nitrosomonas***				0.011	0.011	0.01	0.011	0.011	0.011		0.99	0.15
LGQSHLDPDVPR	***Nitrosomonas***							0.011	0.011	0.011			
VAPMPIAPNPVAIK	***Nitrosomonas***	0.01	0.01	0.01	0.005	0.007	0.009	0.01	0.01	0.01			
FVGGDGIcSGELPK	***Paucibacter***					0.015	0.017	0.018	0.016	0.014		5.32	3.40
NNLSGDLEGGFYVKPTVFK	***Paucibacter***				0.087	0.083	0.083	0.086	0.084	0.082			
STFANVGGGGAK	***Pseudomonas***	0.983			0.983	0.983	0.983	0.983	0.983	0.983		97.97	0.21
GFLTDVEIAK	***Pseudomonas***	0.98	0.98	0.98	0.981	0.979	0.98						
LTDAAPLIAAQESAR	***Pseudomonas***	0.979	0.979	0.979									
VEGDVVGTYLHGNK	***Pseudomonas***	0.981	0.981	0.981									
VQDALEIVGLR	***Pseudomonas***	0.979	0.979	0.979	0.979	0.979	0.979						
TQGLEPGLPTPVIITVYSDR	***Pseudomonas***	0.977	0.977	0.977	0.979	0.977	0.975						
ICAETGVNPAAK	***Pseudomonas***	0.977	0.978	0.978									
TNLQNLNER	***Rheinheimera***				0.964	0.962	0.964	0.962	0.963	0.96		95.54	0.86
DTSFGFGIVQAK	***Rheinheimera***	0.941	0.941	0.941	0.959	0.957	0.954	0.957	0.959	0.969			
VLDNAVADLTAISGQKPLVTK	***Rheinheimera***				0.953	0.951	0.953	0.951	0.953	0.949			
VQALSLTTEGAGNALLQLMNAPR	***Rhodoferax***	0.956	0.957	0.956	0.951	0.948	0.95	0.941	0.944	0.937		92.83	3.19
AAAAAPVAAAAASK	***Rhodoferax***						0.873	0.894	0.89	0.871			
VVANDGQLGGTLGR	***Undibacterium***				0.93	0.925	0.93	0.932	0.93	0.928		94.46	1.09
MHEISDVSIK	***Undibacterium***				0.953	0.949	0.952	0.951	0.951	0.948			
STDFSAILTTIK	***Undibacterium***	0.962	0.962	0.948	0.946	0.95	0.949	0.949	0.946				

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6. Complex food webs coincide with high genetic potential for chemolithoautotrophy in fractured bedrock groundwater

Martina Herrmann, **Patricia Geesink**, Lijuan Yan, Robert Lehmann, Kai Uwe Totsche and Kirsten Küsel

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Groundwater ecosystems face the challenge of energy limitation due to the absence of light-driven primary production. Lack of space and low oxygen availability might further contribute to generally assumed low food web complexity. Chemolithoautotrophy provides additional input of carbon within the subsurface, however, we still do not understand how abundances of chemolithoautotrophs, differences in surface carbon input, and oxygen availability control subsurface food web complexity. Using a molecular approach, we aimed to disentangle the different levels of potential trophic interactions in oligotrophic groundwater along a hillslope setting of alternating mixed carbonate-/siliciclastic bedrock with contrasting hydrochemical conditions and hotspots of chemolithoautotrophy. Across all sites, groundwater harbored diverse protist communities including *Ciliophora, Cercozoa, Centroheliozoa*, and *Amoebozoa* but correlations with hydrochemical parameters were less pronounced for eukaryotes compared to bacteria. *Ciliophora*-affiliated reads dominated the eukaryotic data sets across all sites. DNA-based evidence for the presence of metazoan top predators such as *Cyclopoida* (*Arthropoda*) and *Stenostomidae* (*Platyhelminthes*) was only found at wells where abundances of functional genes associated with chemolithoautotrophy were 10 to 100 times higher compared to wells without indications of these top predators. At wells closer to recharge areas with presumably increased inputs of soil-derived substances and biota, fungi accounted for up to 85 % of the metazoan-curated eukaryotic sequence data, together with a low potential for chemolithoautotrophy. Although we did not directly observe higher organisms, our results point to the existence of complex food webs with several trophic levels in oligotrophic groundwater. Chemolithoautotrophy appears to provide strong support to more complex trophic interactions, feeding in additional biomass produced by light-independent CO_2-fixation.

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Complex food webs coincide with high genetic potential for chemolithoautotrophy in fractured bedrock groundwater

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18S rRNA V1–V3

ABSTRACT

Groundwater ecosystems face the challenge of energy limitation due to the absence of light-driven primary production. Lack of space and low oxygen availability might further contribute to generally assumed low food web complexity. Chemolithoautotrophy provides additional input of carbon within the subsurface, however, we still do not understand how abundances of chemolithoautotrophs, differences in surface carbon input, and oxygen availability control subsurface food web complexity. Using a molecular approach, we aimed to disentangle the different levels of potential trophic interactions in oligotrophic groundwater along a hillslope setting of alternating mixed carbonate-/siliciclastic bedrock with contrasting hydrochemical conditions and hotspots of chemolithoautotrophy. Across all sites, groundwater harbored diverse protist communities including *Ciliophora*, *Cercozoa*, *Centroheliozoa*, and *Amoebozoa* but correlations with hydrochemical parameters were less pronounced for eukaryotes compared to bacteria. *Ciliophora*-affiliated reads dominated the eukaryotic data sets across all sites. DNA-based evidence for the presence of metazoan top predators such as *Cyclopoida* (*Arthropoda*) and *Stenostomidae* (*Platyhelminthes*) was only found at wells where abundances of functional genes associated with chemolithoautotrophy were 10–100 times higher compared to wells without indications of these top predators. At wells closer to recharge areas with presumably increased inputs of soil-derived substances and biota, fungi accounted for up to 85% of the metazoan-curated eukaryotic sequence data, together with a low potential for chemolithoautotrophy. Although we did not directly observe higher organisms, our results point to the existence of complex food webs with several trophic levels in oligotrophic groundwater. Chemolithoautotrophy appears to provide strong support to more complex trophic interactions, feeding in additional biomass produced by light-independent CO_2-fixation.

1. Introduction

An estimated 90% of the prokaryotic biomass on Earth is hidden within the subsurface, hosting mainly microbial communities of bacteria and archaea (Bar-On et al., 2018). But also protozoa, fungi, and metazoa play an active role in subsurface biogeochemical processes (Griebler and Lueders, 2009). In groundwater ecosystems, prokaryotic activity is strongly influenced by nutrient availability supporting both autotrophic and heterotrophic microbial biomass production, contrasting with low intensity of top-down control by higher trophic levels (Foulquier et al., 2011). However, selective grazing of bacterivorous protozoa may result in changes of the bacterial community composition (Novarino et al., 1997; Sinclair et al., 1993; Kinner et al., 2002). Stygofauna – groundwater metazoans – also consume microbial biomass and thus, contribute substantially to water purification and maintenance of hydraulic connectivity, but they can also stimulate microbial processes via excretion of organic substances (reviewed in Saccò et al., 2019). However, their distribution is strongly limited by the availability of food and oxygen (Mösslacher, 2003; Hahn, 2006).

Photosynthesis-driven primary production fuels complex food webs in pelagic freshwater or marine ecosystems with multiple trophic levels, including unicellular organisms up to large metazoa (Brett and Goldman, 1997; D'Alelio et al., 2016). Due to the lack of sunlight and space, and often anoxic conditions in the subsurface, groundwater food webs have been believed to be species poor and

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often to comprise only one or two trophic levels (Gibert and Deharveng, 2002). But near-surface groundwater still receives allochthonous organic matter input with spatial and temporal variation depending on the hydrological connectivity to the surface (Foulquier et al., 2011; Hutchins et al., 2016). Furthermore, there is growing evidence of an important role of chemolithoautotrophic CO_2-fixation in subsurface carbon cycling (Pedersen, 1997; Alfreider et al., 2003; Akob and Küsel, 2011; Herrmann et al., 2015), forming an additional basis of aquifer food webs. In fact, biomass produced by chemolithoautotrophy represents a rather stable resource, since its availability is largely decoupled from surface processes (Hutchins et al., 2016), given a geogenic availability of the preferentially exploited inorganic electron donor. In a recent study of karstic Edwards Aquifer, Hutchins et al. (2016) showed that organic matter which was produced chemolithoautotrophically accounted for 25%–69% of the organic matter utilized by stygobiont communities at higher trophic levels. Similarly, 21% of the diet of cave-adapted shrimps in coastal groundwater of the Yucatan Peninsula was based on carbon from methanotrophic bacteria (Brankovits et al., 2017). However, more detailed information is needed on how and how much chemolithoautotrophy affects eukaryotic species diversity, their feeding strategies, and trophic interactions among all community members along the groundwater food chain. Similarly, we need to know how eukaryotic communities and their interactions are modulated by hydrochemical conditions.

The Hainich Critical Zone Exploratory (CZE), located in central Germany, provides unique subsurface access to study groundwater ecology in sedimentary bedrock along a groundwater monitoring transect under temperate climate conditions. The hillslope flow system in thin-bedded limestone-mudstone alternations is strongly compartmentalized with characteristic biogeochemical regimes from oxic to anoxic conditions (Küsel et al., 2016; Kohlhepp et al., 2017), which allows for the exploration of surface-subsurface interactions and controls of subsurface food webs under different redox regimes. Along the transect, we identified certain hot spots for chemolithoautotrophy (Wegner et al., 2019). Several studies revealed corresponding hydrochemistry-driven patterns of bacterial, archaeal, and fungal diversity in the groundwater (Lazar et al., 2017; Schwab et al., 2017; Nawaz et al., 2018), while communities at higher trophic levels and their potential feeding strategies are largely unknown. In this study, we hypothesized that the number of trophic levels, in particular the presence of metazoan top predators, is primarily determined by oxygen availability in the Hainich oligotrophic groundwater, and that the identity of the food web's key players and their resulting interactions are shaped by the different hydrochemical regimes of the groundwater. Furthermore, we assumed that the differences of chemolithoautotrophic potentials observed along the transect will affect trophic interactions within the subsurface food webs.

2. Methods

2.1. Study site, sampling of groundwater, and chemical analysis

In a central German cultural landscape (NW Thuringia), the monitoring groundwater well transect of the Hainich CZE accesses Triassic bedrock at the eastern hillslope of the Hainich low-mountain range. The sloping Muschelkalk strata comprise thin-bedded marine alternations of limestones and mudstones and host a multi-storey aquifer system (Kohlhepp et al., 2017). The study site and its hydrogeological and hydrochemical properties have been previously described in detail (Küsel et al., 2016; Kohlhepp et al., 2017). In brief, oxic conditions occur in the limestone-dominated main aquifer of the Upper Muschelkalk (Trochitenkalk formation; wells H13, H31, H41, and H51) as well as in uphill zones of the hanging minor and mudstone-rich aquifers (Meissner formation; wells H14, H32). In the downslope direction, both shallow wells (H42, H43) and deep wells (H52, H53) of the minor aquifer assemblage are characterized by suboxic to anoxic conditions (Fig. 1A). Wells also differ with regard to nitrogen chemistry with moderate to high nitrate concentrations in wells H31 and H32 and lower concentrations in wells H13, H14, H42, H43, H52 and H53 (Fig. 1A). The groundwater of all wells is characterized by circumneutral pH (6.9–7.4), high concentrations of dissolved CO_2 and bicarbonate (0.6–1.6 mmol L^{-1} and 5.0–7.7 mmol L^{-1}, respectively), and low but rather steady contents of organic carbon (TOC 1.6–2.0 mg L^{-1}). Concentrations of reduced sulfur compounds (sulfide, thiosulfate) as potential electron donors for autotrophic metabolism are usually below the detection limit of 0.1 μmol L^{-1} at all sites. We collected groundwater in September 2013, July 2014, December 2014, March 2015, June 2015, May 2016, and July 2016 from ten wells in total, with sampling depths ranging from 5 to 88 m below surface (Fig. 1).

Prior to sampling, groundwater was pumped with a submersible sampling pump (MP1, Grundfos, Bjerringbro, Denmark) until stationarity of physicochemical parameters was reached. Specific electrical conductivity, pH, water temperature, dissolved oxygen concentration, and redox potential were measured electrometrically on-site in a flow-through cell with daily calibrated sensor-systems (WTW, Weilheim, Germany). Further chemical analysis was carried out as described previously (Kumar et al., 2017) (see Supplementary Material). For later extraction of DNA and RNA, samples were filtered through 0.2 μm polyethersulfone filters (Supor, Pall Corporation) or 0.2 μm polycarbonate filters (Nuclepore, Whatman), respectively, with 5–6 L of groundwater passing through one filter. Filters were transferred to sterile reaction tubes, frozen on dry ice, and stored at −80 °C until nucleic acid extraction.

2.2. Extraction of nucleic acids, PCR, and amplicon sequencing

Genomic DNA and Total RNA were isolated using the PowerSoil DNA Extraction Kit and the PowerWater RNA Isolation Kit (MOBIO), respectively. For each site, nucleic acids were extracted from one filter per time point. DNase treatment (Turbo DNase free kit, Ambion, USA) and reverse transcription (RT; Array Script Reverse Transcriptase, Ambion) was performed as described in Herrmann et al. (2012).

Bacterial 16S rRNA genes and eukaryotic 18S rRNA genes were sequenced using MiSeq Illumina amplicon sequencing at LGC Genomics GmbH (Berlin, Germany) using the primer combinations Bakt_0341F/Bakt_0785R (Klindworth et al., 2013) and Euk_A7F/Euk516R (Medlin et al., 1988; Kowalchuk et al., 1997), targeting the V3–V4 and the V1–V3 region of the 16S rRNA and 18S rRNA genes, respectively. Sample processing and sequencing have been described elsewhere (Kumar et al., 2017). In addition, we performed MiSeq Illumina amplicon sequencing of genes encoding the large subunit of RubisCO form II (*cbbM*), form IA (*cbbL-IA*), and form IC (*cbbL-IC*), using the primer combinations F-cbbM/R-cbbM, F-cbbL/R-cbbL (Alfreider et al., 2003), and cbbL-1C–F/cbbL-1C-R (Alfreider et al., 2009). All amplifications were performed using Hotstar Mastermix (Qiagen), followed by PCR product purification using NucleoSpin extract II kit (Macherey-Nagel, Germany). Preparation of libraries for MiSeq Illumina amplicon sequencing was carried out using NEBNext Ultra DNA library preparation kit and NEBNext Multiplex Oligos (New England Biolabs), followed by sequencing on a MiSeq sequencing platform using v3 chemistry (Illumina, The Netherlands). Amplicon sequencing of bacterial and eukaryotic SSU rRNA genes was performed for seven time points (September 2013, July 2014, December 2014, March 2015, June

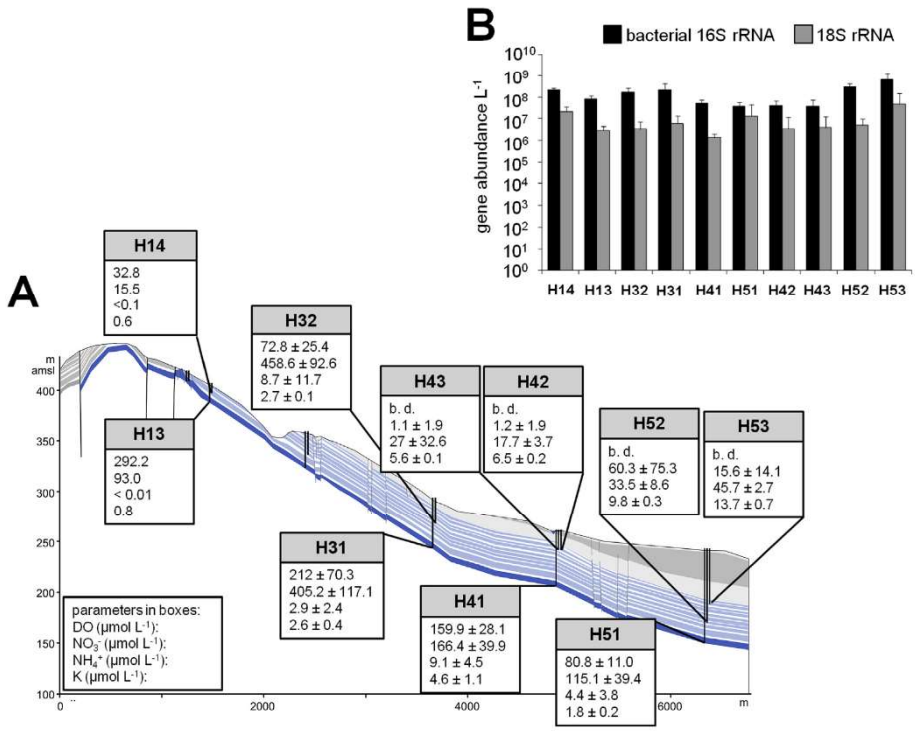

Fig. 1. Location of groundwater wells, hydrochemical characteristics, and microbial abundances based on qPCR analysis across the two aquifer assemblages of the Hainich CZE. (A) Concentration of selected hydrochemical parameters (dissolved oxygen (DO), NO_3^-, NH_4^+, and K). (B) Abundances of bacterial 16S rRNA genes and eukaryotic 18S rRNA genes per L groundwater; mean (±standard deviation) of seven time points per well (H13, H14: two time points; H31: six time points). 18S rRNA gene abundances were corrected for the fraction affiliated with metazoa or plants as revealed by amplicon sequencing. The cross section is taken from Kohlhepp et al. (2017, modified). Groundwater wells are shown as strong vertical black lines. Faults are indicated by thin vertical lines.

2015, May 2016, July 2016), and RubisCO-encoding genes were analyzed for the September 2013 samples. DNA-based analysis was complemented with RNA-based sequencing for samples obtained in July 2014, March 2015, and June 2015 (bacterial 16S rRNA) or March and June 2015 (eukaryotic 18S rRNA). Bacterial 16S rRNA gene sequences obtained in July 2014, May 2016, and July 2016 were already included in previous publications (Schwab et al., 2017; Herrmann et al., 2019). Tests of sequencing consistency including biological and technical replication (filtration, DNA extraction, and amplicon sequencing) performed for groundwater wells H41 and H52 in July 2016 revealed only very small variation between replicates of each site compared to the spatial differences we observed between sampling sites.

2.3. Sequence analysis

Bacterial 16S rRNA gene sequences and eukaryotic 18S rRNA gene sequences obtained from MiSeq Illumina amplicon sequencing were analyzed using Mothur v1.39 (Schloss et al., 2009) along with the SILVA bacteria reference alignment v132 (Quast et al., 2013) according to the Schloss SOP (http://www.mothur.org/wiki/Schloss_SOP; Kozich et al., 2013). Chimera search was done using the uchime algorithm implemented in Mothur. Raw read numbers ranged from 17816 to 267356 (average: 85049) and from 19000 to 318665 (average: 74958) for the eukaryotic and bacterial SSU rRNA data sets, respectively. Read numbers after quality filtering ranged from 3842 to 123272 (average: 41315) and from 9730 to 226296 (average: 40947) for the eukaryotic and bacterial SSU rRNA data sets, respectively (Supplementary Tables 1 and 2). To improve approximation of the relative abundances of unicellular taxa based on the 18S rRNA gene sequence data, we removed all sequences affiliated with plants or metazoa prior to assignment of Operational Taxonomic Units (OTUs) and further statistical analysis. The uncorrected data are provided as Supplementary Material (Supplementary Figs. 1 and 2). Bacterial and eukaryotic community data sets were normalized to the same number of sequence reads per sample (9730 reads for the bacterial communities and 6057 reads for the eukaryotic communities) using the sub. sample command implemented in Mothur, which led to the exclusion of a few samples with read numbers lower than 6057 for the eukaryotic data sets. Sequence analysis of RubisCO encoding genes was carried out using Mothur with a few adjustments necessary for the analysis of protein-encoding genes including BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) as described in Kumar et al. (2017) (see Supplemental Information). Data originating from Illumina MiSeq amplicon sequencing have been submitted to the European Nucleotide Archive under project PRJEB33253, sample accession numbers ERS3548267-ERS3548404.

2.4. Quantitative PCR

Abundances of bacterial 16S rRNA genes, eukaryotic 18S rRNA genes, and protein-encoding genes involved in CO_2-fixation were assessed using quantitative PCR (qPCR) with Brilliant II SYBR Green qPCR Mastermix (Agilent Technologies) on a Mx3000P instrument

(Agilent Technologies). Bacterial 16S rRNA gene and eukaryotic 18S rRNA genes were amplified using the primer combination Bac8F-mod/Bac338abc (Loy et al., 2002; Nercessian et al., 2005) and Euk-A7-F/Euk-516R (Medlin et al., 1988; Kowalchuk et al., 1997), respectively. qPCR of RubisCO-encoding genes was performed using the same primer combinations as used for MiSeq Illumina amplicon sequencing. Abundances of *nxrB* genes encoding nitrite oxido-reductase of *Nitrospira* and *hzsA* genes encoding hydrazine synthase of bacteria performing anaerobic ammonia oxidation (anammox) were determined using the primer combination nxrB169F/nxrB638R (Pester et al., 2013) and hzsA1597F/hzsA1857R (Harhangi et al., 2012).

2.5. Statistical analysis

All statistical analyses were performed using the software PAST (Hammer et al., 2001). More detailed information is provided as Supplementary Material. Co-occurrence networks were calculated using the online tool MENA (Molecular Ecological Network Analysis Pipeline, http://ieg4.rccc.ou.edu/mena/login.cgi) (Deng et al., 2012) with settings as described in Supplementary Material. Networks were visualized using Cytoscape v. 3.4.1.

3. Results

3.1. Groundwater harbors diverse communities of protists, fungi, and metazoa

To address the question if eukaryotic and bacterial community patterns and abundances show similar preferences across the different depths and topographic positions, and thus groundwater quality, we compared bacterial and eukaryotic community structures for seven time points on the DNA-level. In addition, three and two time points were analyzed on the RNA-level for bacteria and eukaryotes, respectively. Using a 0.03 distance cut-off for the assignment of species-level OTUs, numbers of OTUs ranged from 297 to 4701 (average: 1134) for eukaryotic 18S rRNA genes (without metazoa) and from 749 to 4456 (average: 2177) for bacterial 16S rRNA genes (Supplementary Tables 1 and 2). Based on non-metric multidimensional scaling, clustering patterns were less distinct for the eukaryotic compared to the bacterial communities (Supplementary Fig. 3) using Jaccard similarity indices of OTU distribution patterns across samples. For both communities, samples from wells H13/H14 at the hilltop position, and samples from wells H42/H43 and H52/H53, respectively, clustered separately. However, temporal variation among samples taken from the same well was higher for the eukaryotic communities.

Abundances of eukaryotic 18S rRNA genes originating from protists and fungi varied by two orders of magnitude across sites and ranged from 1.6×10^5 to 4.8×10^7 genes per L groundwater. In contrast, abundances of bacterial 16S rRNA genes showed lower spatial variations by only one order of magnitude with additionally rather stable abundances over time at each individual groundwater well (Fig. 1B). Maximum bacterial 16S rRNA gene abundances were observed at the two suboxic to anoxic wells H52 and H53 (mean values 2.4×10^8–3.5×10^8 genes per L groundwater), and lower abundances in the anoxic groundwater of wells H42 and H43 (2.2×10^7 and $1.5\ 10^7$ genes L^{-1}), and in the oxic groundwater of the two downstream wells H41 (4.0×10^7 genes L^{-1}) and H51 (2.8×10^7 genes L^{-1}).

Across all sites, we found a strong predominance of few species-level taxa within the eukaryotic communities with the 15 most abundant OTUs accounting for 17–59% of the 18S rRNA gene sequence reads (Fig. 2). In general, *Ciliophora* formed the largest fraction of the eukaryotic sequence reads (18–81%) with the highest contribution in the anoxic groundwater of wells H52/H53, followed by *Cercozoa* with the highest fraction in the oxic wells H31, H32, H41, H51 (mean values 5.2–48%). A large part of this fraction was often accounted for by a single species-level OTU, such as EukOtu03 (*Cercozoa*: 40% of the sequence reads in oxic well H41) or EukOtu02 (*Ciliophora*: 33–39% in wells H52 and H53; Fig. 2B). Other flagellate groups commonly observed in the groundwater included *Ochrophyta*, *Aquavolonida*, *Ichthyosporea*, and *Protalveolata* (Fig. 2). Sequences affiliated with *Centroheliozoa*, *Amoebozoa* (*Tubulinea* and *Discosea*) and *Pigoraptor* were consistently detected in small fractions of usually less than 4% in both oxic and anoxic groundwater.

The largest read fraction of metazoan origin was affiliated with *Chaetonotidae* (*Gastrotricha*), which were found in both oxic and anoxic groundwater (up to 53% of the non-corrected data set; Supplementary Figs. 1 and 2). Groundwater of wells of the lower oxic aquifer (main aquifer), except for the hilltop location, occasionally harbored signals of the crustacean group *Cyclopoida* (*Maxillopoda*: 0.1–5.1%), which were also found in lower read numbers in the anoxic groundwater of well H52. Similarly, sequences affiliated with *Stenostomidae* (0.1–11.8%), freshwater catenulid flatworms belonging to the Platyhelminthes, were detected under both oxic and anoxic conditions in wells H31, H32, H41, H51, and H52. Sequences affiliated with these metazoan top predators were detected in 74% of the samples from oxic wells and, in turn, were absent from 75% of the anoxic wells, pointing to a positive relationship between presence of metazoan top predators and oxic conditions (Chi-Square test, $p = 0.0009$). Sequences affiliated with

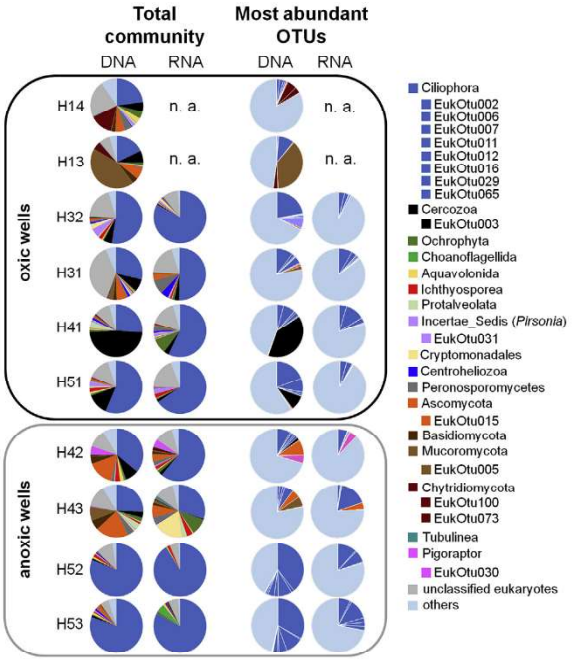

Fig. 2. Eukaryotic community structure in the groundwater of ten wells of the two aquifer assemblages. Charts represent mean values of seven time points on the DNA-level or two time points on the RNA level. Left panel: Community structure on the phylum level (class level where appropriate). Right panel: Mean relative abundances of the 16 most abundant OTUs across sites. No RNA-based information is available for sites H13 and H14 (n. a.). Sequence reads affiliated with metazoa or plants were removed from the data sets. Samples with fractions of Ascomycota >90% (see Fig. 3) were excluded from the calculation of mean values.

Rotifera (*Ploimida*) were only found in the groundwater of wells H31, H32, H41, H51, H42 and H43 (Supplemental Figs. 1 and 2). *Porifera* accounted for up to 3.3% of the sequence reads in the anoxic wells H42 and H43 but for less than 0.6% across the other wells. In turn, sequence reads affiliated with subterranean *Polychaeta* (*Troglochaetus beranecki*; 99.8% sequence identity) were occasionally observed in the oxic wells but were not detected in the anoxic wells. Except for the two wells at the hilltop position of the transect, all wells also harbored sequence reads affiliated with *Cnidaria*, distantly related to *Antipatharia* (*Anthozoa*; 88% sequence identity). Occasionally, we also found signatures of *Nematoda* (wells H14, H43, H52, H53) or of soil-dwelling animals such as *Collembola* (wells H14, H31, H32, H42, H51). In addition, we detected low numbers of sequence reads of other metazoa (usually < 10 reads in a few samples) affiliated with *Malacostraca*, *Lumbriculida*, *Aranea*, *Acari*, and *Insecta*, and higher plants, most likely originating from pollen.

Notably, for the samples obtained in March 2015 and June 2015, up to 99.9% of the 18S rRNA gene sequence reads were affiliated with *Ascomycota* (*Hypocreales*) for six of the eight wells sampled at that time point (Fig. 3). These increased fractions of *Ascomycota* coincided with an increase in 18S rRNA gene abundances by up to one order of magnitude, suggesting that these fungi occurred sporadically in large amounts in addition to the groundwater protist and fungi community observed at the other time points (Fig. 3). While *Ascomycota* were also commonly detected in the

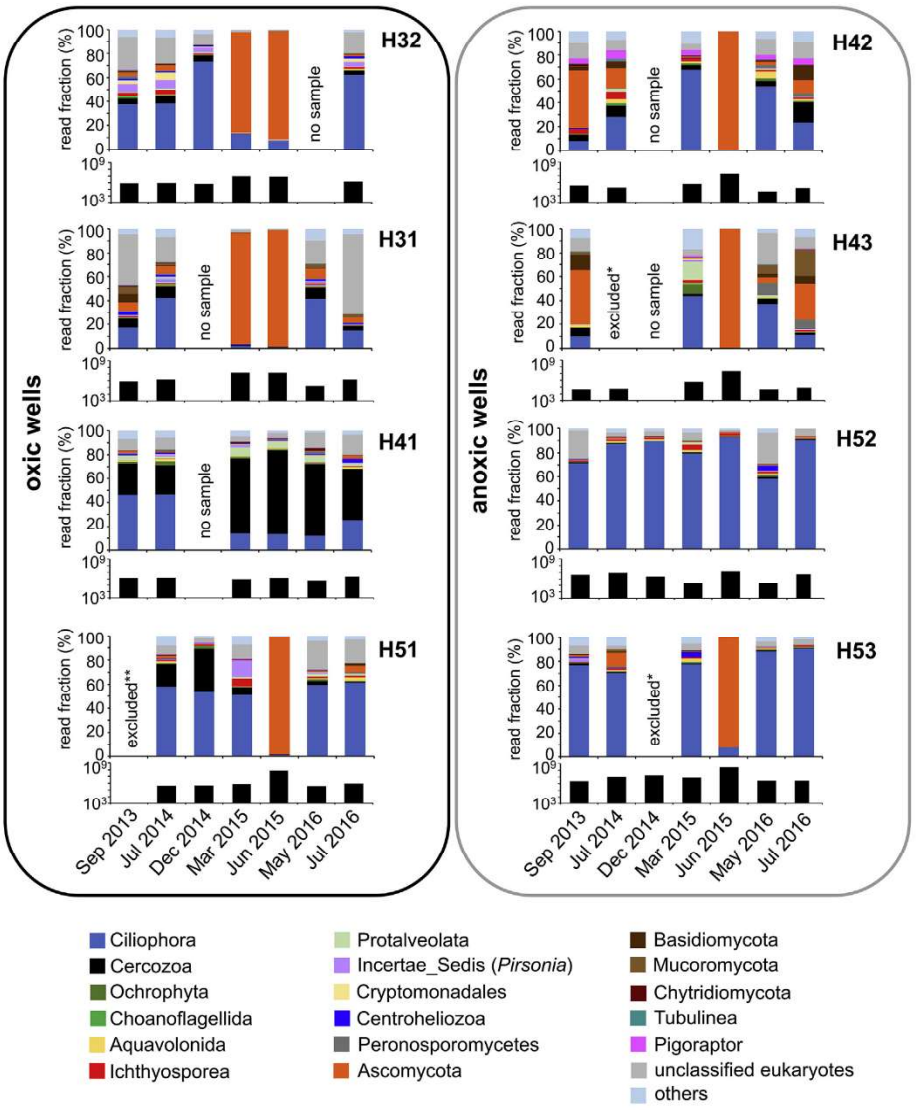

Fig. 3. Temporal changes in the community structure of eukaryotic microorganisms in eight groundwater wells across six time points (stacked bars;H52 : seven time points) and in the abundance of 18S rRNA genes per L groundwater (black bars). Sequence reads affiliated with metazoa or plants were removed from the data sets, and qPCR data were corrected for the corresponding fraction. Results of wells H13 and H14 are not shown as only data from two time points are available. *excluded because sequence read number was lower than threshold for subsampling (6057 reads); **excluded because of potential contamination with *insecta*-affiliated DNA.

groundwater of wells H42 and H43 (mean values 15.9–19.9% of the sequence reads; Fig. 2), they formed only a small fraction of the eukaryotic reads in the groundwater of the other wells (0.3–7.3%). In wells H13 and H14 at the hilltop position, we found an increased fraction of *Mucoromycota* (EukOtu05 accounting for 38% of the sequence reads in well H13), *Chytridiomycota*, and smaller contributions of *Basidiomycota* (Figs. 2 and 3). Despite their occasional predominance in the DNA-based communities, *Ascomycota* were only poorly represented in the communities derived from RNA-based sequencing. The strongest indication of the presence of active fungi was obtained for wells H31, H42, and H43, where fungal reads accounted for 6.8–11.5% of the sequences of the RNA-based fraction. Across all sites, the RNA-based fraction was mostly dominated by *Ciliophora*-affiliated reads (Fig. 2). Among metazoan groups, *Cyclopoida, Chaetonotidae,* and *Rotifera* were also detected on the RNA-level in selected wells (Supplementary Fig. 1).

Out of the 30 most abundant protist or fungal OTUs, a considerable fraction did not show any correlation with 15 hydrochemical parameters tested in this study (Supplementary Fig. 4). For some eukaryotic groups, dissolved oxygen or the parameter-combination sodium, magnesium, ammonium, and potassium played an important role in distinguishing sub-communities that corresponded in a similar way to these parameters. DOC and TOC were hardly involved in significant correlations. Since the availability of prey may be an important driver of the distribution patterns of eukaryotic taxa, we correlated estimated total abundances of the 140 most abundant eukaryotic OTUs with bacterial 16S rRNA gene abundances. Among the protist groups, 45% of all *Ciliophora*-affiliated OTUs and 60% of *Centroheliozoa*- or *Aquavolonida*-affiliated OTUs in this subset were positively correlated to bacterial abundances while this fraction was lower for other protist groups (Supplementary Table 3). An overview of the taxonomic affiliation and the fasta-sequences of all OTUs is provided as Supplementary Material (Supplementary data set "Euk_taxonomy.xls"; Supplementary data sets "rep_Otus_protists_fungi.fasta", "rep_Otus_metazoa_plants.fasta").

3.2. *Cand.* Patescibacteria and Nitrospirae dominate the bacterial communities

In contrast to the eukaryotic taxa, the 30 most abundant bacterial species-level taxa appeared to have specific preferences for a given hydrochemical environment (Supplementary Fig. 5), suggesting that the mechanisms driving bacterial and eukaryotic

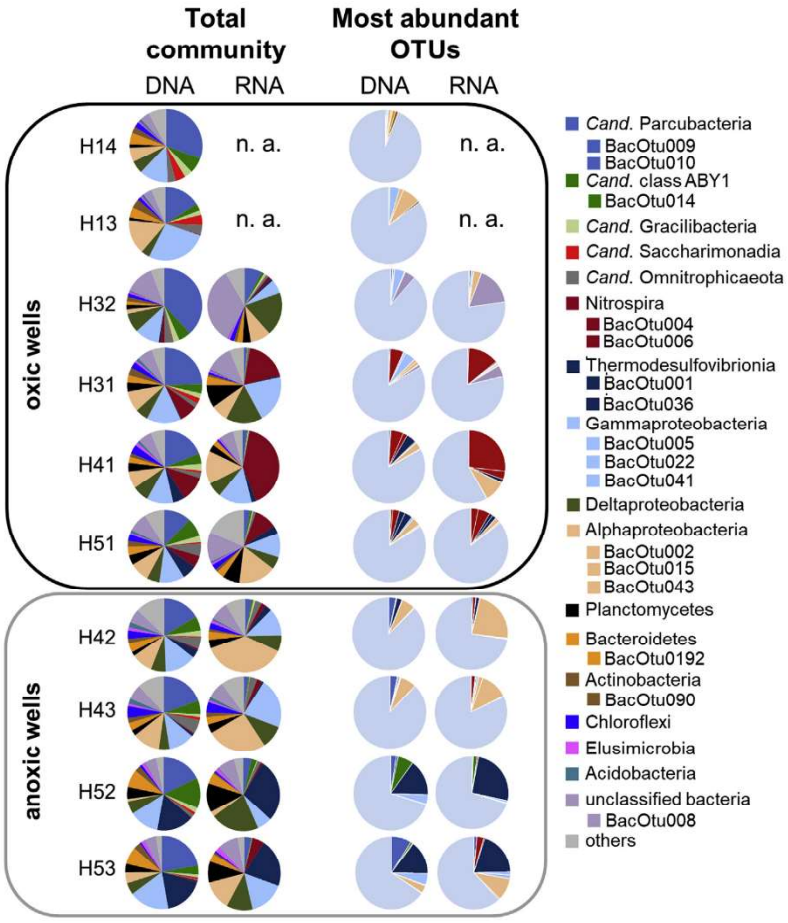

Fig. 4. Bacterial community structure in the groundwater of ten wells of the two aquifer assemblages. Charts represent mean values of seven time points on the DNA-level or three time points on the RNA level. Left panel: Community structure on the phylum level (class level where appropriate). Right panel: Mean relative abundances of the 16 most abundant OTUs across sites. No RNA-based information is available for sites H13 and H14 (n. a.).

distribution patterns across the hillslope groundwater flow system were substantially different. In general, groundwater bacterial communities as potential prey for the eukaryotic taxa were dominated by members of the *Cand.* Patescibacteria, *Nitrospirae*, *Gamma-* and *Deltaproteobacteria*, which together accounted for 50–70% of the total community across sites and time points. Candidate phyla including *Cand.* Patescibacteria (*Cand.* Parcubacteria, *Cand.* class ABY1, *Cand.* Gracilibacteria, *Cand.* Saccharimonadia) and *Cand.* Omnitrophicaeota were especially abundant in the groundwater communities of oxic wells H14 and H32 with a contribution of almost 50% (Fig. 4A). Contrasting with their predominance on the DNA-level, candidate phyla were strongly underrepresented in the bacterial communities derived from RNA-based sequencing. Here, dominance was shifted in favor of *Thermodesulfovibrionia* (*Nitrospirae*) distantly related to the presumably facultative autotroph "*Cand.* Nitrobium" (Arshad et al., 2017), *Gamma-* and *Deltaproteobacteria* and autotrophic *Brocadiaceae* (*Planctomycetes*) in wells H52 and H53. A single OTU affiliated with *Thermodesulfovibrionia* (BacOtu001) already accounted for 15% and 26% of the sequence reads on the DNA and RNA level, respectively. In the oxic groundwater of wells H31, H41, and H51, sequence reads affiliated with the genus *Nitrospira* (*Nitrospirae*) were strongly increased in the RNA-based communities (up to 41% in well H41 with two OTUs BacOtu004 and BacOtu006 already accounting for 31%), which could be either autotrophic nitrite oxidizers (Lücker et al., 2010) or autotrophic comammox bacteria capable of complete ammonia oxidation to nitrate (Van Kessel et al., 2015; Daims et al., 2015). An overview of the taxonomic affiliation and the fasta-sequences of all bacterial OTUs are provided as Supplementary Material (Supplementary data set "Bac_taxonomy.xls"; Supplementary data set "rep_Otus_bac.fasta").

3.3. Abundance and composition of key autotrophic bacterial communities

The high abundance of presumably autotrophic community members (*Nitrospira*, *Thermodesulfovibrionia*, *Brocadiaceae*) was further confirmed by high abundances of *nxrB* genes encoding nitrite oxido-reductase of *Nitrospira* (H31: 6.3×10^6 L^{-1}; H41: 1.4×10^6 L^{-1}), or *hzsA* genes encoding hydrazine synthase of anammox bacteria (*Brocadiaceae*) (H52: 4.7×10^7 L^{-1}; H53: 4.6×10^7 L^{-1}) in the respective wells (Fig. 5). To get more insight into autotrophic microbial groups using the Calvin-Benson-Bassham cycle (CBB) that were previously identified as ubiquitous at our study site (Wegner et al., 2019), we targeted *cbbM*, *cbbL* (IA) and *cbbL* (IC) genes encoding RubisCO form II, IA, and IC, respectively. Taking together the information from all functional genes tested, the highest potential for CO_2-fixation was found in the groundwater of wells H52 and H53 followed by wells H31, H32, H41. Ten to 100 times lower abundances of the respective genes were found in the groundwater of wells H42 and H43 and at the hilltop location of the transect (wells H13, H14) (Fig. 5).

Also the sum of all three RubisCO encoding genes was highest in the anoxic wells H52 and H53 (5.8×10^6–1.3×10^8 genes L^{-1} groundwater) along with a maximum fraction of *cbbM* genes within the RubisCO encoding gene pool. *cbbM* or *cbbL* (IA) genes were mainly affiliated with sulfide, thiosulfate, or sulfur oxidizers related to *Sulfuricaulis limicola*, *Sulfurifustis variabilis*, *Sulfuricella denitrificans*, *Thiobacillus thiophilus*, *T. thioparus*, and *Cand.* Thiodictyon syntrophicum, followed by organisms capable of oxidation of reduced sulfur and hydrogen, such as *Sulfuritalea hydrogenivorans* (Supplemental Figs. 6A and B). In addition, the communities harboring *cbbL* (IA) and *cbbL* (IC) genes were dominated by potential hydrogen oxidizers such as *Hydrogenophaga* sp. at most sites, while ammonia oxidizers affiliated with *Nitrosomonas* sp. and *Nitrosospira* sp. formed a large fraction of the community encoding *cbbL* (IC) genes in well H41 (Supplemental Fig. 6C).

3.4. Co-occurrence networks and inferred trophic interactions

Various interactions between microorganisms such as competition, mutualism, or predation may lead to co-occurrence or mutual exclusion of organisms, resulting in distinct distribution patterns of sub-communities. To generate hypotheses about the potential underlying ecological mechanisms, we used co-occurrence network analysis of originally 2261 bacterial and 480 eukaryotic OTUs (protists and fungi), which resulted in a network with significant positive and negative interactions between 730 bacterial and 94 eukaryotic members, represented as nodes in the network (Supplementary Fig. 7). 24% of the eukaryotic OTUs had 5

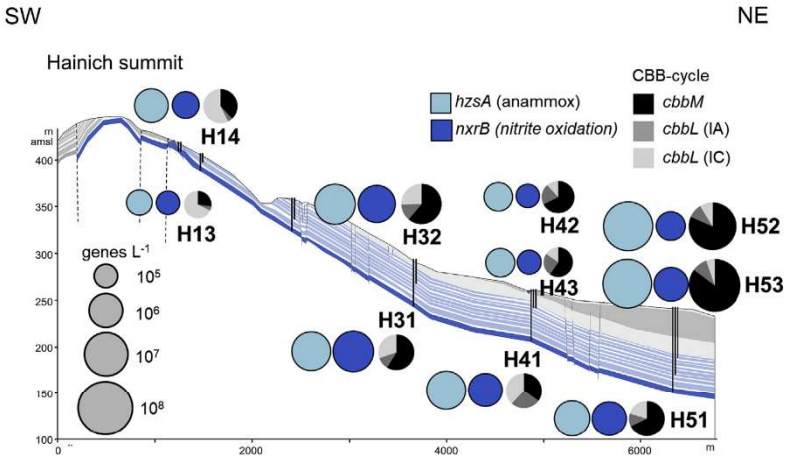

Fig. 5. Abundance of *nxrB* genes encoding nitrite oxido-reductase (*Nitrospira*), *hzsA* genes encoding hydrazine synthase (anammox bacteria), and RubisCO-encoding genes involved in the Calvin- Benson-Bassham cycle (CBB-cycle). Relative fractions of *cbbM*, *cbbL* (IA) and *cbbL* (IC) genes were normalized to the sum of these three genes, data represent means of six to seven time points (two for H13 and H14). Size of the circles indicates the sum of all three RubisCO- encoding genes or *nxrB* and *hzsA* genes, respectively, as gene abundances per L groundwater. The cross section is taken from Kohlhepp et al. (2017, modified).

or more links to other OTUs (maximum 34 links), indicating that protist and fungal species were highly interconnected in the groundwater microbial community. The highest number of connections were found for representatives of *Tubulinea* (EukOtu000402), *Cyclidium* (EukOtu000290, *Ciliophora*), and *Perkinsidae* (EukOtu000046, *Protalveolata*). Negative interactions between bacterial and protist OTUs as an indicator of predator-prey-relationships (Chow et al., 2014), were observed for some members of the *Cercozoa*, *Ichthyosporea*, *Cryptomonadales*, *Tubulinea*, *Choanoflagellida*, and *Protalveolata*. In contrast, members of the *Ciliophora* mostly exhibited positive interactions with bacterial OTUs. Notably, we also found frequent negative connections among eukaryotic OTUs, such as for *Cercozoa*, *Tubulinea*, *Pigoraptor*, *Cryptomonadales*, and *Prostomatea* (*Ciliophora*). Across all bacterial OTUs involved in negative connections with eukaryotes, taxonomic affiliation reflected the most abundant bacterial taxa in the groundwater including *Cand.* Patescibacteria, *Proteobacteria*, and *Bacteroidetes*.

To summarize our key findings, we constructed a simplified conceptual model, assigning the different eukaryotic taxa to postulated feeding strategies and different levels in the trophic cascade (top predators, first- and second-level-predators, primary consumers including heterotrophic bacteria and fungi, and chemolithoautotrophic bacteria as primary producers) (Fig. 6). Evidence of metazoan top predators such as *Cyclopoida* and *Platyhelminthes* was restricted to the groundwater of wells H31, H32, H41, H51, H52 and H53, resulting in more complex food webs for these wells compared to the hilltop position of the transect (wells H13/H14) and the anoxic wells H42/H43. Representatives of first-level predators (*Cercozoa*, *Chaetonotidae*, *Ichthyosporea*, and

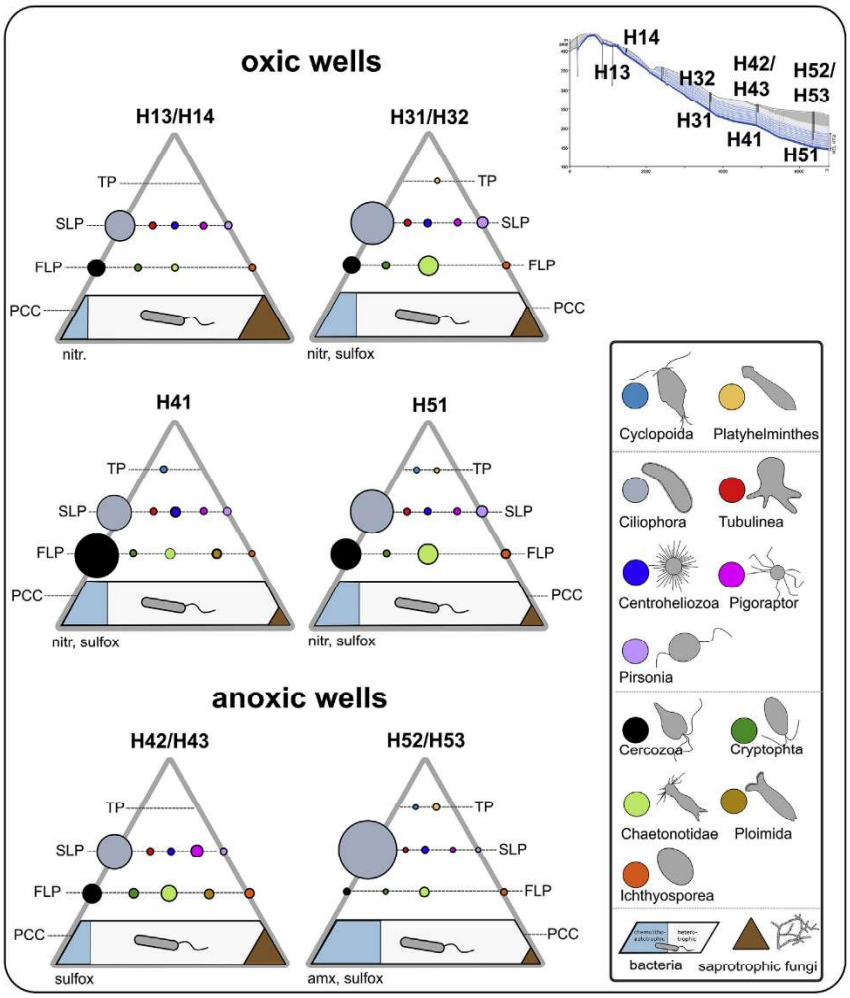

Fig. 6. Simplified conceptual model of the trophic interactions in the groundwater of the Hainich CZE, integrating five different trophic levels - chemolithoautotrophy and primary consumers (PCC; bacteria and fungi), first level predators (FLP), second level predators (SLP), and metazoan top predators (TP). Most abundant taxa representative of the different trophic levels are indicated by different colors. Sizes of circles represent mean sequence read fractions associated with a certain taxonomic group (for chemo-lithoautotrophic bacteria: estimated fraction within the total bacterial community based on qPCR data (see Fig. 5) and 16S rRNA gene-based amplicon sequencing; see Supplementary Table 2). Most important metabolic processes providing energy for CO_2-fixation are indicated (nitr = nitrification; sulfox = oxidation of reduced sulfur compounds, amx = anammox). For wells with similar eukaryotic community structure, information was combined (H13/H14; H31/H32; H42/H43; H52/H53). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Cryptophyta) and second-level predators (*Tubulinea, Centroheliozoa, Pigoraptor*, and *Pirsonia*) were detected across all sites based on the molecular data. Species affiliated with *Ciliophora* as the most abundant protist group represented both first-level and second-level predators. The bacterial community fraction with the genetic potential for chemolithoautotrophy was estimated from the 16S rRNA- and functional gene-based analysis, and mean values per site ranged from 0.8 to 36% with the highest potential for chemolithoautotrophy in the groundwater of wells H52/H53 and the lowest potential at the hilltop location (wells H13/H14; Supplementary Table 4). Sequence-based evidence of the presence of metazoan top predators (*Cyclopoida*, *Stenostomidae*) was significantly linked to sites where the genetic potential for chemolithoautotrophy was 10% or more of the bacterial community (Chi-Square test, $p = 0.0024$). Thus, our data suggest that increased complexity of trophic levels that included these top predators coincided with a high estimated genetic potential for chemolithoautotrophy. Moreover, we observed opposing trends between the potential for chemolithoautotrophy and the fraction of fungi-affiliated sequence reads, i.e., low genetic potential for chemolithoautotrophy coincided with a high fraction of fungal reads within the 18S rRNA gene data sets (wells H13/H14 and H42/H43), and vice versa (wells H52/H53) (Spearman rank correlation coefficient −0.534, $p = 0.0003$).

4. Discussion

We tested the hypothesis that the number of trophic levels in oligotrophic groundwater, including invertebrates, is primarily determined by oxygen availability. We also expected trophic interactions and their key players to be shaped by the heterogeneity of hydrochemical conditions which is characteristic for the different habitats in a hillslope flow system. Indeed, we found hydrochemistry-driven distribution patterns of all groundwater organisms but patterns were stronger for bacterial than for eukaryotic communities. Bacteria depend directly on the availability of organic or inorganic electron donors, while protists and metazoa often have a broad prey spectrum, which might explain the weaker linkage to specific hydrochemical settings.

The subterranean food web structure paradigm assumed pervasive generalist feeding strategies, low trophic complexity, and short food chains (Gibert and Deharveng, 2002; Hutchins et al., 2016). This assumption of generalist feeding was recently challenged by Francois et al. (2015), demonstrating a high degree of trophic specialization for two isopod species obtained from caves. Here, we collected molecular evidence for a high diversity of eukaryotic microorganisms in oligotrophic groundwater, forming a complex trophic cascade based on their taxonomic affiliation and inferred feeding strategies. Previously, mostly flagellates morphospecies (<10 μm) of sessile or free-living suspension feeders were identified in groundwater from a drinking water well accessing Upper Muschelkalk bedrock strata (Risse-Buhl et al., 2013) like the groundwater monitoring transect of the Hainich CZE. Now we were able to provide evidence from molecular analysis for the presence of larger protists and even detect signals of metazoa, as wells of the groundwater monitoring transect were specifically designed to also sample large organisms and particles by using coarse slotted well screens and coarse annulus backfill instead of filter sand in the main screen sections (Küsel et al., 2016).

Inferred trophic interactions and their key players differed substantially across sites. We found molecular evidence of the presence of metazoan top predators with the highest metazoan read fractions in oxic groundwater, likely regardless of lithostratigraphic affiliation. Here, a combination of oxic conditions and space given by fractures, especially in the Trochitenkalk main aquifer might have supported a high diversity of protists and the presence of larger metazoa with a broad range of feeding strategies and body size up to a few millimeters. Metazoan taxa can act as end members of the groundwater trophic cascade, e. g., *Cyclopoida* and *Platyhelminthes*, which are common members of groundwater habitats (Deharveng et al., 2009), feed also on protozoan and metazoan prey (Morselli et al., 1998; Brandl, 2005), while others contribute directly to the control of bacterial biomass (e. g., *Chaetonotidae*, *Rotifera*, *Porifera*; Bennett, 1979; Simoes et al., 2013; Manconi and Pronzato, 2015). No molecular evidence for metazoan top predators was found in anoxic, even very shallow wells H42/H43, while, surprisingly, we detected sequences affiliated with these organisms in anoxic deep wells H52/H53. Oxygen concentrations around 0.5–1 mg L^{-1} were thought as critical thresholds for stygofauna occurrence (Hahn, 2006), which is still higher than the concentrations we measure in these wells (Kohlhepp et al., 2017). Many groundwater metazoa are known to tolerate hypoxic conditions for some time (Malard and Hervant, 1999) but they are not known to thrive without oxygen for longer times. However, the molecular signature of an organism cannot be equaled by the presence of a live organism because sequences may also originate from pieces of tissue or animal remains. Nonetheless, sequences were found at the deep anoxic wells H52/H53 that are known to have less surface connectivity than the shallow anoxic wells H42/H43 where these sequences were not detected. Hence, direct sampling of larger metazoa, which needs special technical equipment, is needed to shed more light on the presence of metazoa under conditions of low oxygen availability.

We also detected sequences affiliated with *Cnidaria* distantly related to *Antipatharia* (*Anthozoa*) across almost all sites. In marine environments, these organisms act as suspension feeders but are also capable of feeding on larger metazoa (Wagner et al., 2012). Nonetheless, given the low sequence identity, we did not assign these *Cnidaria* to a top predator position in the groundwater food web.

On the level of unicellular eukaryotes, we observed a low but constant fraction of sequence reads affiliated with *Amoebozoa* (*Tubulinea* and *Discosea*), *Centroheliozoa*, and *Pigoraptor* across all sites, which could act as first-level or second-level predators. *Amoebozoa* feed on bacteria, fungi, or smaller protists, and therefore serve as important linkages in food webs between microbes and higher organisms (Anderson, 2017; Ren et al., 2018). They can encyst and excyst relatively rapidly (Anderson, 2017), which could facilitate their dispersal from soil to the groundwater and enable their persistence within the aquifers. Surface attachment is needed for naked amoeba while feeding on prey (Anderson, 2017), suggesting that microorganisms colonizing surfaces of the aquifer rock are an important component of the aquifer food web. Recently, we identified the bacterial communities colonizing rock fracture surfaces of rock material obtained during drilling. Those bacteria are distinct from the ones living within the rock matrix, and bacteria colonizing rock surfaces range from organic matter decomposers in outcrop areas to chemolithoautotrophs in downdip positions (Lazar et al., 2019). Thus, surface attached *Amoebozoa* might feed on a broad spectrum of different bacterial taxa and functional groups depending on the local aquifer architecture and other habitat conditions.

Similar to *Amoebozoa*, *Centrohelida* and heliozoan-like protists feed on bacteria, other protists, and larvae of invertebrates under preferentially oxic conditions (Gast, 2017), which was reflected by the highest sequence read fraction affiliated with these organisms in the oxic groundwater of midslope wells H31, H32 and H41. Although *Heliozoa* are known as predators of the free water column, they have frequently been observed in microscopy-based studies of groundwater (Novarino et al., 1997; Loquay et al.,

2009), and could be enriched from groundwater of our site (unpublished results). Further trophic complexity was added by molecular evidence for the presence of protists distantly related to the recently described *Pigoraptor*, a unicellular predator that feeds on eukaryotic prey (Hehenberger et al., 2017), and to the genus *Pirsonia* known as parasitoid nano-flagellates (Kühn et al., 2004).

Among all protist groups, *Ciliophora* appeared to be the most successful and competitive based on their representation in the sequence data sets on both the DNA and RNA level. *Ciliophora* can act both as primary and secondary predators (Fenchel, 2013). Their predominance across all sites might be due to their broad spectrum of feeding strategies and body sizes, as well as to the ability of many ciliates to cope with microoxic conditions or live anaerobically (Dubber and Gray, 2011). Only at sites with higher oxygen availability (wells H31, H32, H41 and H51), other bacterivorous protists such as *Cercomonas* (*Cercozoa*) and *Goniomonas* (*Cryptomonadales*) made up a larger fraction of the eukaryotic sequence data sets, suggesting that they could successfully compete with ciliates for bacterial prey.

Our molecular findings of ciliate predominance are in contrast with previous microscopic assessments (Novarino et al., 1997). Given the fact that 18S rRNA operon numbers per ciliate cell may often exceed 1000 or 10,000 (Gong et al., 2013), we cannot rule out that the 18S rRNA sequence data sets are biased towards *Ciliophora* or the predominance of a few *Ciliophora*-affiliated community members. In fact, numbers of *Ciliophora* individuals could be much lower than the sequence read fractions suggest. Consequently, quantitative relationships between the different eukaryotic taxa cannot be resolved with the molecular data alone, and the observed predominance of *Ciliophora*-affiliated sequence reads needs to be interpreted with caution.

The presence of the predatory surface sliders *Litonotus* and *Loxophyllum* (Fenchel, 2013) points again to rock surface contact as important lifestyle for some groundwater *Ciliophora*. These protists seem to follow their bacterial prey more often than controlling its abundance, as shown by the positive correlation of 45% of the most abundant *Ciliophora*-OTUs with bacterial 16S rRNA gene abundances. Similar relationships were previously observed for virus-bacteria-interactions (Chow et al., 2014). Compared to viral lysis, grazing can be more influenced by size rather than taxonomy of the prey (González et al., 1990; Hahn and Höfle, 1999). In marine systems, grazing by protozoa is thought to target primarily larger cells (Fuhrman, 2009). Consequently, small-sized cells belonging to the candidate phyla radiation (Brown et al., 2015; Luef et al., 2015) should be less affected by grazing and establish and maintain larger populations. However, *Cand*. Patescibacteria were equally frequent in negative interactions with protist OTUs in the microbial network, as were *Proteobacteria* or *Bacteroidetes*, suggesting that they might be equally affected by grazing.

As diverse stygobiont communities in fractured and karstic aquifers or oligotrophic subterranean environments can be supported by biomass produced by chemolithoautotrophy (Hutchins et al., 2016; Brankovits et al., 2017), we aimed to link our findings on eukaryotic diversity and trophic interactions to the bacterial potential for chemolithoautotrophy as the basis of the groundwater food web. Overall, we found striking coincidence of a high genetic potential for chemolithoautotrophy and a high complexity of the groundwater food web in terms of molecular evidence for the presence of metazoan top predators, which applied to both oxic (H31, H32, H41) and anoxic conditions (H52, H53). Chemolithoautotrophic potentials were mainly linked to nitrification at site H41, a spatial zone characterized by nitrification (Opitz et al., 2014; Wegner et al., 2019), and to anammox performed by *Brocadiaceae* in the anoxic environments of wells H52 and H53 (Starke et al., 2017; Schwab et al., 2017; Kumar et al., 2017; Wegner et al., 2019). Here, anammox bacteria occurred together with species-level taxa affiliated with the poorly characterized class *Thermodesulfovibrionia* (*Nitrospirae*), which could also play a role in CO_2-fixation using the Wood-Ljungdahl pathway linked to nitrogen or sulfur cycling (Arshad et al., 2017). Maximum abundances of RubisCO-encoding *cbbM* genes in these wells provided further support of a large chemolithoautotrophic community driven by the oxidation of reduced nitrogen and sulfur compounds, and hydrogen. As slow growing bacterial populations are discussed to be more affected by grazing protists (Sinclair and Alexander, 1989), especially chemolithoautotrophic denitrifier or anammox bacteria populations that are known to have doubling times in the range of days to weeks (Kumar et al., 2018; Strous et al., 1999) should be effectively reduced. However, given the limited quantitative information provided by the eukaryotic molecular data, potential top-down control mechanisms on chemolithoautotrophs remain currently unclear.

The highest fraction of sequence reads affiliated with fungal groups, like *Mucoromycota*, *Chytridiomycota*, and *Ascomycota* were found at the hilltop locations (H13, H14) and shallow midslope positions (H42, H43) where seepage may rapidly percolate through the soil cover. These findings point to a direct import of fungi from surface soils or to fungal populations feeding on soil derived organic matter available at these sites. Here, we also found a generally low genetic potential for autotrophic CO_2 fixation, which might underline allochthonous carbon supply. A high fraction of fungal signatures affiliated with *Hypocreales* (EukOtu001), which are saprotrophic, parasitic or pathogenic *Ascomycota*, temporarily appeared in some groundwater wells in March and June 2015. For most of these sites, Nawaz et al. (2018) observed a similar shift towards *Ascomycota* within the active fungal communities at the same time points. This temporal *Ascomycota* dominance may point to an event-driven import from surface soils, probably as fungal spores, mediated by hydraulic events such as snowmelts or precipitation (Griebler and Lueders, 2009; Dibbern et al., 2014; Küsel et al., 2016). Surface-derived inputs may lead to temporary perturbations of the groundwater trophic interactions by an enhanced import of soil-borne microorganisms, especially of fungi, and an increased availability of allochthonous organic carbon.

5. Conclusions

- Our molecular findings suggest the existence of complex food webs in the groundwater of fractured sedimentary bedrock with several levels along the trophic cascade up to metazoan top predators such as *Cyclopoida* or *Platyhelminthes*.
- We found strong evidence for a high genetic potential for chemolithoautotrophy of up to 36% of the bacterial community, probably supporting complex groundwater food webs under both oxic and anoxic conditions. Chemolithoautotrophy was energetically linked to nitrification, anammox, and the oxidation of reduced sulfur compounds and hydrogen.
- Our sequence data clearly showed the omnipresence of *Ciliophora* across the different groundwater wells, most likely linked to their high versatility regarding oxygen requirements, feeding strategies, body size, and prey spectrum.
- Taken together our study suggests a substantial input of carbon entering complex aquifer food webs via light-independent CO_2-fixation.

Author contributions

MH and KK designed this study. KT and KK designed the Hainich Critical Zone Exploratory, and RL is responsible for field

infrastructure. MH and PG performed the molecular work, and MH performed the sequence analysis. KT and RL provided hydrochemical data. LY contributed to the network analysis. MH wrote the manuscript with contributions from all other authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.115306.

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7. General Discussion

Invisible in our everyday live, the subsurface is often overlooked as a compartment that provides important services for humans (Ford and Williams, 2007). However, the terrestrial subsurface hosts different environments, including groundwater, that are of great importance for sustaining life above the ground. Groundwater stored in karst systems is one of the main resources for drinking and service water worldwide. Thus, understanding the chemical, physical and biological processes that take place in this complex environment is key to perceive the significance of natural and anthropogenic influences in this important ecosystem. In order to understand the relevance of biological processes within the subsurface, a detailed understanding of the origin of dominant microorganisms, their metabolic potential and the effects of interactions between microorganisms is needed.

7.1 Interactions of Groundwater Microorganisms

7.1.1 Interactions via Secondary Metabolites

Screening bacterial isolates for their ability to produce secondary metabolites that affect the growth of other microorganisms has classically been done on solid agar plates. Additionally, the screened bacteria were often obtained from easy to access environments, like soils. Within the framework of this dissertation, a high-throughput screening method for both growth-inhibiting and promoting secondary metabolites produced in liquid cultures, has been established (Geesink *et al.*, 2018; **Chapter 2**). This method allows to automatically screen thousands of mono- and co-cultures in liquid media, saves time and avoids user bias compared to previously developed, not-automated methods for bacteria growing on solid medium (Tyc *et al.*, 2014). The possibility to detect growth-promoting effects adds significant value to this screening method (Geesink *et al.*, 2018; **Chapter 2**). By that, not only new antimicrobial compounds, but also novel growth promoting substances for organic fertilizers can potentially be discovered using this screening technique.

Compared to other environments like soils, cell densities in groundwater are orders of magnitudes lower and available nutrients are scarce (Akob and Küsel, 2011; Herrmann *et al.*, 2019; **Chapter 3**). Thus, we hypothesized, that cooperative interactions leading to growth promotion of neighboring organisms should prevail within the groundwater community.

However, among the tested 149 bacterial isolates, negative interactions dominated (Geesink *et al.*, 2018; **Chapter 2**). On the one hand, the cooperative exchange of metabolites with unspecific partners in an environment with low abundances might against our assumptions not be an effective strategy. On the other hand, the investigated bacterial isolates only represent a small fraction of the bacterial diversity in groundwater and cooperative interactions potentially dominate within the yet uncultured part of the groundwater microbiome (Geesink *et al.*, 2018; **Chapter 2**).

Furthermore, the co-existence of different microorganisms can enhance the formation of metabolites that in turn affect the growth of a third organism. For soil ecosystems various studies show, that co-cultivation of bacterial isolates increases the formation of growth affecting metabolites (Garbeva *et al.*, 2011; Traxler *et al.*, 2013; Kinkel *et al.*, 2014; Tyc *et al.*, 2014, 2017). However, co-cultivation of bacteria isolated from groundwater, although they generally show a high potential for the production of growth-influencing secondary metabolites, does neither increase growth promoting nor inhibiting effects (Geesink *et al.*, 2018; **Chapter 2**). Direct competition for nutrients and space within a co-culture can prevent bacteria from investing in the production of secondary metabolites (Geesink *et al.*, 2018; **Chapter 2**). Unlike in soil, where bacteria are present in proximity, groundwater microorganisms might be more adapted to a "dilute" environment. Low cell numbers and restricted formation of biofilms due to the oligotrophic conditions in the groundwater, presumably limit the probability of direct cell-cell contact in the aquifer. Thus, the development of mechanisms that trigger the formation of secondary metabolites in the presence of other bacteria might not be an effective strategy for bacteria in oligotrophic groundwater (Geesink *et al.*, 2018; **Chapter 2**).

The high potential of growth-influencing secondary metabolites production by individual members of the screened microbial community demonstrates the impact that these bacteria can have on the performance of other microorganisms in the environment. At the same time, the bacterial isolates that were tested only represent a small fraction (3.52 %) of the actual groundwater microbiome and belonging to the phyla *Proteobacteria*, *Actinobacteria*, *Bacteriodetes* and *Firmicutes,* that have been shown to be the dominating microorganisms in soils, while their relative abundance in the groundwater decreases (Herrmann *et al.*, 2019; **Chapter 3**). In contrast, members belonging to phyla that for the most part lack cultivated representatives, including bacteria of the CPR, are flourishing in groundwater (Luef *et al.*, 2015; Herrmann *et al.*, 2019; **Chapter 3**). In order to assess potential interactions of these organisms, detailed analysis of their metabolic potential based on available genomes, as well as innovative attempts to improve cultivation strategies are needed.

7.1.2 Potential Symbionts within the Candidate Phyla Radiation

Bacteria belonging to the CPR are typically characterized by small, streamlined genomes and are thus depending on partner organisms (Luef *et al.*, 2015; Castelle *et al.*, 2018; Cross *et al.*, 2019; Geesink *et al.*, 2019; **Chapter 4**). Members of the CPR have been found to be dominant members of groundwater ecosystems (Luef *et al.*, 2015; Hubalek *et al.*, 2016; Schwab *et al.*, 2017; Proctor *et al.*, 2018). As cultivated representatives of the CPR are rare, genome-centric approaches can enable the elucidation of potential interactions with other microorganisms and even more so, the mechanisms behind these interactions (Geesink *et al.*, 2019; **Chapter 4**). However, little is known about how these microorganisms become dominant members of the groundwater microbiome and how these high abundances are linked to potential partner organisms. The design of the Hainich CZE (**Figure 3**) enables the sampling of ground- and seepage water as well as soil in the groundwater recharge area and thus allows to track inputs of soil-derived microorganisms into the groundwater. An analysis across these three compartments demonstrated that CPR are potentially being introduced to the groundwater from soils via seepage water (Herrmann *et al.*, 2019; **Chapter 3**). In fact, a similar mobilization of CPR has been observed in agricultural soils (Zhang *et al.*, 2018). Within the groundwater, differences in hydrochemistry and the abundances of putative partner organisms have been found to be the potential driving factors for the abundance of different taxa within the superphylum *Cand.* Patescibacteria (Herrmann *et al.*, 2019; **Chapter 3**).

In order to explain the specific mechanisms that result in a distinct distribution of members of the CPR in a complex aquifer system, we coupled the analysis of abundance data in spatio-temporal resolution of one CPR bacterium, *Cand.* Roizmanbacterium ADI133, with a detailed analysis of its genome (Geesink *et al.*, 2019; **Chapter 4**). Within the typically small and streamlined genome of this organism, multiple host association and virulence factors point towards a symbiotic, potentially parasitic, lifestyle. *Cand.* Roizmanbacterium ADI133 can potentially attach to partner cells using a trimeric autotransporter adhesin and subsequently inhibit their cell wall biosynthesis using a toxin-antitoxin system (Geesink *et al.*, 2019; **Chapter 4**). While a symbiotic lifestyle has been proposed for members of the CPR before, concrete mechanisms have not been described before (Luef *et al.*, 2015; Castelle *et al.*, 2018).

To deduce potential partner organisms of *Cand.* Roizmanbacterium ADI133, we analyzed co-occurrence patterns of the CPR organism with other members of the Hainich groundwater microbiome. Explicit spatio-temporal co-occurrence patterns allowed us to pinpoint one potential partner, a *Thermodesulfovibrionia* (Geesink *et al.*, 2019; **Chapter 4**). However, co-

occurrence patterns within the entire groundwater community have previously shown, that CPR to be frequently co-occurring with hetero- and autotrophic members of the microbial community, as well as other members of the CPR, suggesting that co-occurrence patterns presumably point towards similar ecological preferences (Herrmann *et al.*, 2019; **Chapter 3**).

Closely related species to the potential partner OTU in enrichment cultures (Arshad *et al.*, 2017), as well as genomic data from the Hainich site (Herrmann *et al.*, personal communication) point towards the ability of performing dissimilatory nitrate reduction to ammonium as well as sulfate reduction by the *Thermodesulfovibrionia* (**Figure 5**). Both, nitrogen and sulfur cycling play an important role in the Hainich groundwater, as demonstrated in independent studies (Kumar *et al.*, 2017; Schwab *et al.*, 2017; Wegner *et al.*, 2019). By stimulating the growth of its partner with lactate, *Cand.* Roizmanbacterium ADI133 could potentially have a positive impact on *Thermodesulfovibrionia* and thus indirectly on both sulfur and nitrogen cycling in the groundwater of the Hainich CZE (**Figure 5**).

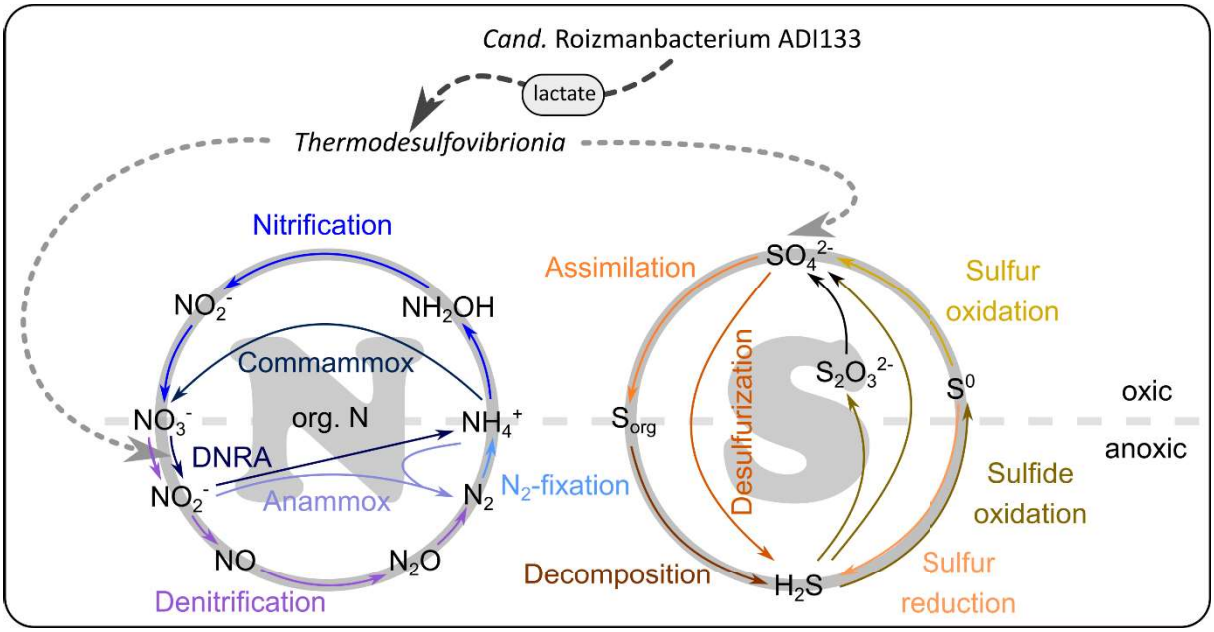

Figure 5 | By transferring lactate to potential partner bacteria *Cand.* Roizmanbacterium could impact biogeochemical cycling of Nitrogen and Sulfur in groundwater of the Hainich CZE.
Cand. Roizmanbacterium ADI133 could provide lactate to its potential partner *Thermodesulfovibrionia* (Nitrospirae), a mixotrophic groundwater microorganism which can perform dissimilatory nitrate reduction to ammonium (DNRA) as well as sulfate reduction. The different processes in nitrogen and sulfur cycling are depicted in different colors, modified after Canfield *et al.* (2010) and Drews (2015).

While an in-depth analysis of the genome of one abundant member of the CPR, as well as the analysis of the distribution and co-occurrence pattern of all CPR present in the studied groundwater system (Herrmann *et al.,* 2019; **Chapter 3**; Geesink *et al.*, 2019; **Chapter 4**), give first insights into potential interactions and the metabolic potential of CPR in groundwater, their role in the ecosystem in large parts remains a "black box". In order to confidently make assumptions about the partner organisms of *Cand.* Roizmanbacterium or other members of the CPR in the Hainich groundwater, novel isolation strategies as presented by Cross and colleagues (2019) are needed.

Collectively, these findings only partially confirm the first hypothesis of this thesis:

Positive interactions between microorganisms prevail in groundwater ecosystems

While we were able to confirm a high potential for interactions between cultured as well as yet uncultured microorganisms in groundwater, the predominance of positive interactions could not be confirmed with the methods applied in this thesis. However, cultivation independent approaches point towards a potential for cooperative interactions by yet uncultivated members of the CPR in the groundwater microbiome.

At the same time the second hypothesis of this thesis was confirmed:

Fermentative members of the CPR are linked to autotrophic partners in the groundwater microbiome

In fact, we were able to show that members of the CPR are frequently positively correlated with autotrophic members of the groundwater microbiome. The example of *Cand.* Roizmanbacterium ADI133 gives further insights into potential associations of CPR with mixotrophic community members, like *Thermodesulfovibrionia*. However, further investigations are needed to confirm and characterize these interactions.

7.1.3 Cultivation-bias and Novel Cultivation Techniques

Although the dogma that only around 1 % of microbes are culturable (Amann *et al.*, 1995) has been challenged (Martiny, 2019), to date, the majority of microbes is still not yet cultured (Steen *et al.*, 2019). The phenomenon of the "uncultured majority" has been first described almost a century ago by Razumov (1932) and was later referred to as the "Great Plate Count Anomaly" (Staley and Konopka, 1985). This gap between diversity estimates as well became obvious when comparing the diversity covered by cultivation approaches, 16S rRNA amplicon sequencing and genome-resolved metagenomics from groundwater of the Hainich CZE (**Figure 6**). Recently, within the domains of Archaea and Bacteria, entire phyla that lack cultivated representatives have recently been discovered that can currently only be detected using cultivation-independent approaches (Brown *et al.*, 2015; Spang *et al.*, 2015; Anantharaman *et al.*, 2016). While cultivation-independent methods can improve our understanding of the metabolic potential of yet uncultured organisms, cultivation-based studies are needed to complement and verify genomic-based hypotheses.

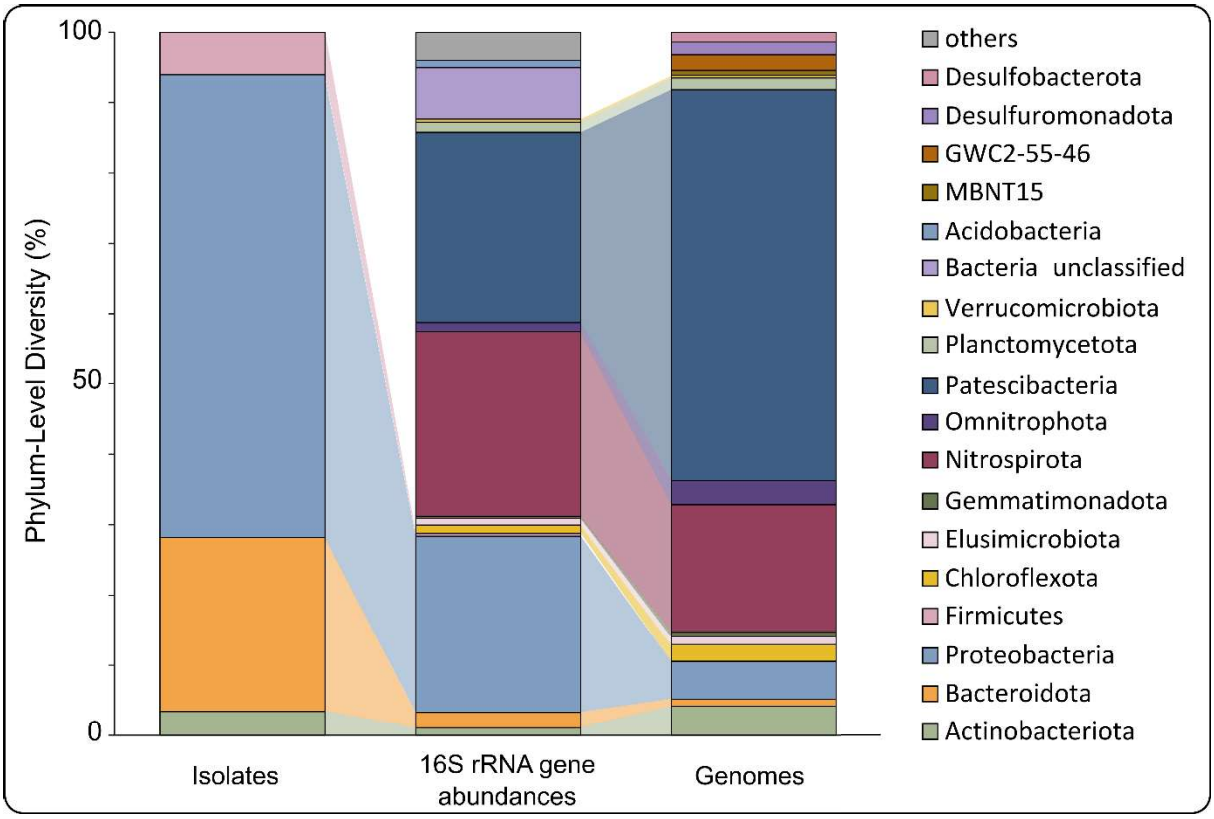

Figure 6 | Comparison of the bacterial diversity in groundwater of the Hainich CZE. Diversity of the isolated bacteria from groundwater (Geesink *et al.*, 2018; **Chapter 2**), the bacterial community estimated via 16S rRNA gene amplicon sequencing (Geesink *et al.*, 2019; **Chapter 4**) and genome-resolved metagenomics (Overholt *et al.*, 2019).

Within the framework of this dissertation, 149 bacterial isolates from groundwater have been obtained. In sum, these isolates account for up to 3.52 % of the bacterial community in the Hainich Critical Zone Exploratory (Geesink *et al.*, 2018; **Chapter 2**). Considering that only a small fraction of the community in an environment can be isolated with classical approaches (Amann *et al.*, 1995; Steen *et al.*, 2019), the isolates obtained from the Hainich CZE cover a remarkable fraction of the groundwater community. However, the microbial diversity revealed by amplicon-based and genome-resolved sequencing approaches exceeds the diversity among the cultured bacteria from our groundwater system (**Figure 6**). Beyond the work conducted within the framework of this dissertation, the isolated bacteria were used to investigate the effects of functional and phylogenetic diversity on synthetic community functioning (Xenophontos *et al.*, 2019) as well as to develop a method to track active microbial cells using single cell Raman micro-spectroscopy (Taubert *et al.*, 2018).

Novel cultivation techniques, like a directed co-isolation of specific microorganisms using techniques like reverse genomics (Cross *et al.*, 2019) could facilitate the cultivation of a broader range of organisms, and potentially also enable the co-isolation of members of the CPR from groundwater with their potential partners. Cross and colleagues (2019) demonstrated for *Cand.* Saccharibacteria, that an isolation of CPR with their hosts is possible, thus, presenting a promising approach also for the groundwater community. Using genome-derived information about specific surface proteins could for example also be used to actively sort and cultivate *Cand.* Roizmanbacterium ADI133 together with its partner. Furthermore, methods like droplet microfluidics can be used to improve the cultivation of for example slow growing microorganisms that are not as competitive under laboratory conditions (Mahler *et al.*, 2015; Kaminski *et al.*, 2016). By separating single cells into individual droplets, competition for nutrients and space gets limited, which enhances the growth of microorganisms that would not be targeted by classical cultivation approaches. Here, information obtained from a detailed analysis of CPR genomes can be used to tailor future cultivation attempts towards individual organisms, like cellulose or necromass that are potential carbon sources of *Cand*. Roizmanbacterium ADI133 (Geesink *et al.*, 2019; **Chapter 4**)

First incubations of groundwater microorganisms in microfluidics show, that even though members of the phyla *Proteobacteria* and *Bacteroidets* are dominating the incubations in droplets, *Cand.* Patescibacteria (CPR) are maintained in the droplet incubations (Geesink and Mahler *et al.*, unpublished; **Figure 7**). Further refinements of the incubation approaches, such as incubations under anoxic conditions, encapsulating single groundwater bacteria with potential partner organisms, or the separation of droplets with lower cell densities

(potentially CPR) from droplets with high cell densities (potentially heterotrophs) are promising strategies to facilitate the cultivation of yet uncultivated microorganisms (Geesink and Mahler *et al.*, unpublished).

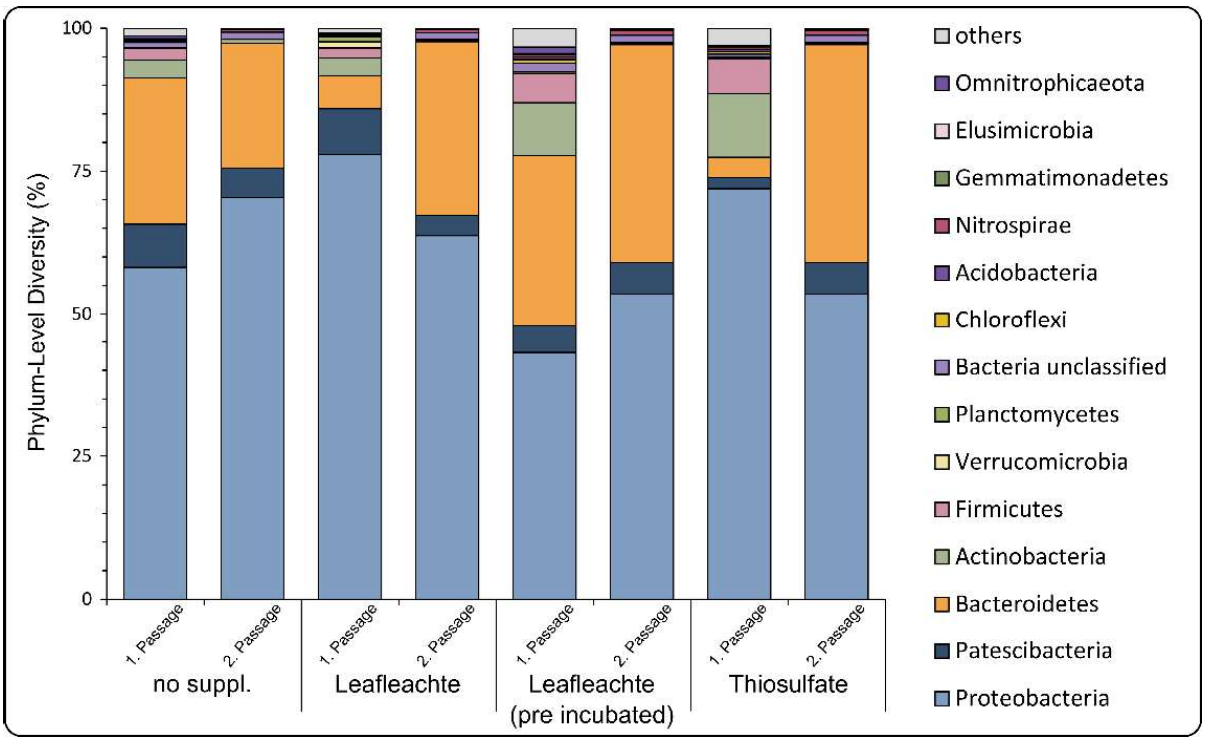

Figure 7 | Innovative cultivation techniques facilitate the cultivation of yet uncultivated bacteria. Relative abundances of bacteria that were maintained in microfluidic droplet incubations over a time span of 7 weeks and two droplet passages (transfers) (Geesink and Mahler *et al.*, unpublished).

7.2 Efficient Recycling of Carbon Supports the Groundwater Microbiome

7.2.1 Reduction of Cell Size in Oligotrophic Environments

One characteristic features of members of the CPR, but also other microorganisms in oligotrophic environments is the reduced cell- and genome size (Hood and MacDonell, 1987; Rocap *et al.*, 2003; Giovannoni *et al.*, 2005; Luef *et al.*, 2015). Up to 54 % of the bacteria in the groundwater of the Hainich CZE have been found to pass through 0.2 µm pore size filters (Herrmann *et al.*, 2019; **Chapter 3**). These bacteria are partially organisms known to have small cell sizes, like members of the CPR, as well as other heterotrophic and autotrophic bacteria that typically have larger cells (**Figure 8A**). Generally, microbial cell size is limited by the space that essential parts of a cell, such as nucleic acids, ribosomes and enzymes require within the cell envelope (Velimirov, 2001). Based on this, a minimum cell diameter between 0.14 µm and 0.30 µm has been proposed (Koch, 1996; Velimirov, 2001; Luef *et al.*, 2015).

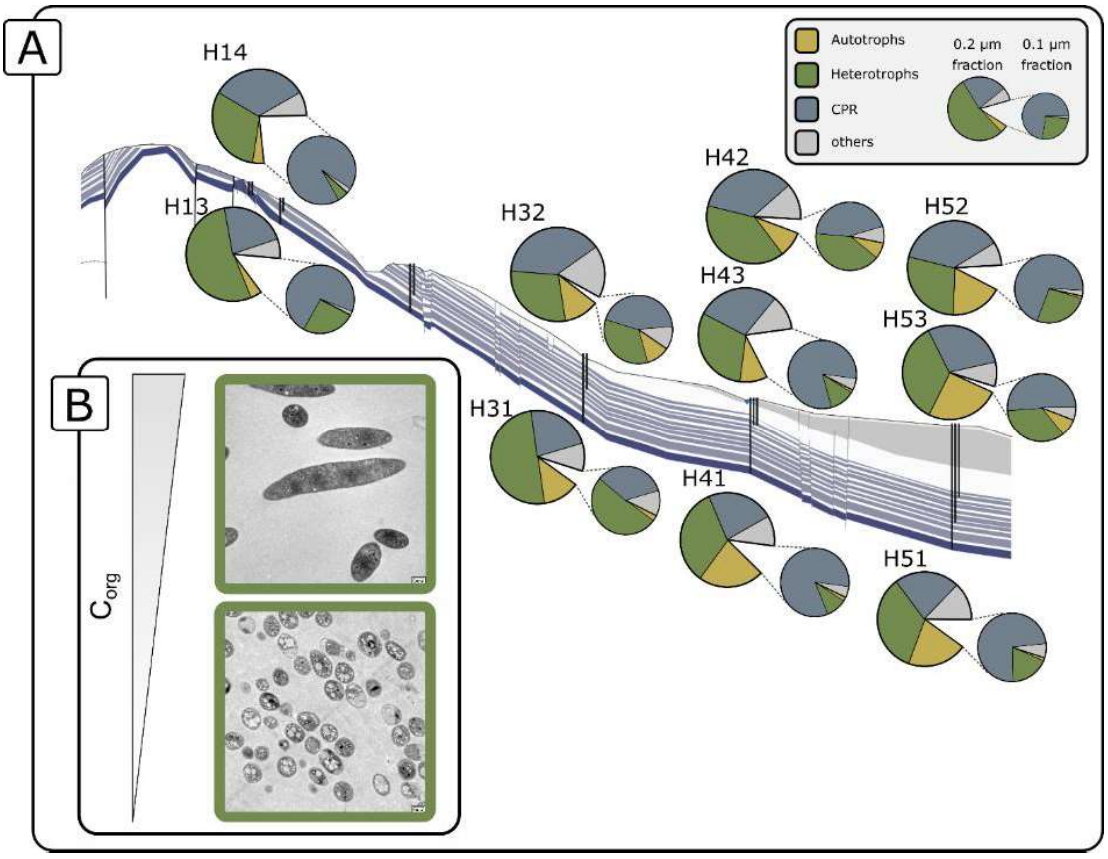

Figure 8 | Variations in cell sizes in different groups of bacteria across the Hainich CZE. (A) The bacterial community within both filter fractions can be differentiated in auto- (yellow) and heterotrophic (green) bacteria, including CPR (blue). (B) A drastic decrease in cell size induced by starvation has been demonstrated for multiple heterotrophic bacteria that were isolated from the Hainich CZE. TEM pictures of a *Flavobacterium* illustrate these effects (Herrmann *et al.*, 2019; **Chapter 3**).

Studies from various environments, report the presence of ultra-small bacterial cells that pass through the commonly used 0.2 µm filters. These studies include terrestrial and marine samples, kidney stones or human cavities (Bae *et al.*, 1972; McKay *et al.*, 1996; Kajander *et al.*, 1998; Miyoshi *et al.*, 2005; Soro *et al.*, 2014; Luef *et al.*, 2015; Herrmann *et al.*, 2019; **Chapter 3**). However, the underlying mechanisms that cause ultra-small cell sizes can be different.

Genome streamlining. Luef and colleagues (2015) highlight the presence of ultra-small bacteria belonging to the CPR in groundwater, whose small cell size below 0.2 µm is related to a small genome size of around 1 Mbp. With that, the genomes of members of the CPR are comparably small, if not smaller, than genomes of other ultra-small bacteria (**Table 1**). The drastic reduction of genome size correlates with reduced metabolic costs for replication (Giovannoni *et al.*, 2014). While other ultra-small bacteria like *Prochlorococcus* are free-living organisms, members of the CPR are assumed to be obligate symbionts. The drastic reduction of their genome led to the loss of essential metabolic features like the ability to synthesize nucleotides, amino acids, vitamins or lipids (Wrighton *et al.*, 2012; Brown *et al.*, 2015; Castelle and Banfield, 2018). First isolates and enrichment cultures confirm the partner-dependent lifestyle of individual members of the CPR (Gong *et al.*, 2014; He *et al.*, 2015; Cross *et al.*, 2019). Likewise, a detailed analysis of the spatio-temporal distribution of CPR member, *Cand.* Roizmanbacterium ADI133, suggests that this bacterium is at least temporarily living attached to other bacteria. The identified mechanisms for attachment to potential partner cells affirm these findings (Geesink *et al.*, 2019; **Chapter 4**).

In marine environments, multiple examples of free-living ultra-small show that streamlined genomes are not leading to an obligate partner association (**Table 1**). Indeed, differences in preferences of being in the 0.2 µm filter fraction despite the presumably smaller cell sizes points towards an attached lifestyle (*Cand.* Roizmanbacteria, candidate class ABY1 *Cand.* Gracilibacteria), whereas other members of the CPR that are enriched in the 0.1 µm fraction might be preferentially free-living (*Cand.* Paceibacteria) (Geesink *et al.*, 2019; **Chapter 4**; Herrmann *et al.*, 2019; **Chapter 3**).

Table 1 | Examples of free-living ultra-small bacteria with reduced genome sizes demonstrate, that genome streamlining does not necessarily lead to an obligate partner association.

Organism	Genome Size	Environment	Reference
Prochlorococcus	1.66-2.41 Mbp	Marine	Rocap *et al.* (2003)
SAR11	1.28-1.46 Mbp	Marine	Giovannoni *et al.* (2005)
OM43	1.30 Mbp	Marine	Giovannoni *et al.* (2008)
Cyanobacterium UCYN-A	1.44 Mbp	Marine	Tripp *et al.* (2010)

An obligate reduction of cell size to lower metabolic costs is only one strategy to persist in oligotrophic environments like groundwater, and beside CPR bacteria, also other heterotrophic bacteria can be found in the 0.1 µm fraction (**Figure 8A**).

Starvation. The persistence of microorganisms in groundwater is strongly linked to their ability to tolerate stress due to the lack of nutrients or variations in nutrient availability (Haller *et al.*, 2000). While the input of organic carbon (OC) to subsurface environments is limited, heterotrophic bacteria depend on this input, otherwise leading to starvation.

We investigated the impact of carbon limitation on 26 representative bacterial isolates from the Hainich CZE and found that 80 % of these heterotrophic organisms reduced their cell size with decreasing availability of OC in the growth medium. Transmission Electron Microscopy of a selected isolate belonging to the genus *Flavobacterium sp.* confirmed a drastic reduction of the cell size and morphology from long, rod-shaped cells to small cocci under conditions of starvation (**Figure 8B**). These results demonstrate how heterotrophic bacteria can adapt to the extreme conditions in groundwater by drastically reducing their cell size. The increase in surface-to-volume ratio improves the uptake of nutrients (Sowell *et al.*, 2009) and thus is beneficial under conditions of carbon starvation (Herrmann *et al*., 2019; **Chapter 3**).

Similarly, marine habitats are often limited in nutrients and have been described to harbour large fractions of ultra-small bacteria (Macdonell and Hood, 1982; Li and Dickie, 1985; Hood and MacDonell, 1987; Haller *et al.*, 2000). Ultra-small bacteria of the genus *Vibrio,* isolated from estuary water, have been shown to reduce their cell size under nutrient limited conditions (Hood and MacDonell, 1987; Nyström *et al.*, 1992). *Flavobacterium* and different Proteobacteria were similarly found to be present in the starved, ultra-small fraction of bacteria in seawater from the Western Mediterranean Sea (Haller *et al.*, 2000). While the effect of OC availability on a subset of members of the groundwater community is striking (Herrmann *et al*., 2019.; **Chapter 3**), implications of in-situ generated as well as recycled OC on the composition and functioning of the entire groundwater microbiome need to be addressed further.

7.2.2 Carbon Flow in Groundwater Food Webs

Chemolithoautotrophy. While microorganisms in the groundwater are well adapted to the conditions in the subsurface by streamlining their genomes or reducing their cell size in phases of starvation, the availability of organic carbon is a strong limiting factor for growth of these organisms. Due to the lack of light-driven primary production and little carbon inputs from the surface, chemolithoautotrophic primary production is of great importance for the groundwater microbiome and fuels subsurface food webs (Akob and Küsel, 2011; Hutchins *et al.*, 2016; Herrmann *et al.*, 2020; **Chapter 6**).

Investigating subsurface environments, that are generally not easy to access, is challenging and studies disentangling microbial food webs in groundwater are limited and have been focussing on cave environments or exclusively targeted the prokaryotic community in the groundwater (Graening and Brown, 2003; Simon *et al.*, 2003). This unidirectional investigation of groundwater as a habitat has often led to the assumption that subterranean food chains are short and the diversity, especially of eukaryotic life, is low (Graening and Brown, 2003; Simon *et al.*, 2003). Contrastingly, other studies found that groundwater harbours complex food webs and a high diversity of all domains of life (Danielopol *et al.*, 2000; Risse-Buhl *et al.*, 2013). This diversity, that is supposedly comparable to the diversity in surface ecosystems, must be supported by an independent energy source (Por, 2007; Hutchins *et al*., 2016). In fact, Por (2007) speculates about the existence of a globally distributed, contiguous biome that is exclusively based on chemolithoautotrophic primary production.

With up to 36% of the bacterial community being chemolithoautotrophic bacteria, the potential of in-situ primary production of biomass in the groundwater of the Hainich CZE is immense. This pool of bacterial biomass supports the complex food webs observed within this groundwater system, independent of surface organic matter input (Herrmann *et al.*, 2020; **Chapter 6; Figure 9**). As a result, the studied aquifers harbour complex food webs that comprise multiple trophic levels, including *Ciliophora*, as first- and second-level predators. Their high versatility with respect to oxygen tolerance, feeding strategies as well as cell size, might allow *Ciliophora* to inhabit various micro-habitats within the complex aquifer system. Furthermore, molecular evidence for the presence of metazoan top predators such as *Cyclopoida* or *Platyhelminthes* was found (Herrmann *et al.*, 2020; **Chapter 6**). While the presence of diverse protozoa has been confirmed by cultivation- and microscopy based investigations of the aquifer system (Risse-Buhl *et al.*, 2013), the presence of metazoan organisms in the aquifer system should be confirmed likewise.

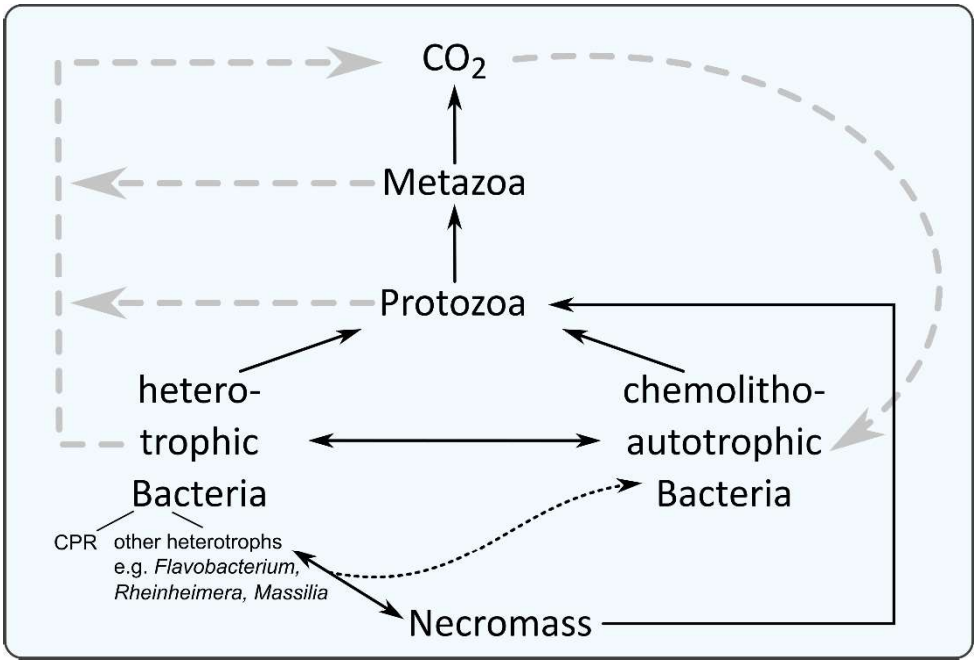

Figure 9 | Proposed trophic interactions within the groundwater microbiome. Complex food webs in karst aquifer systems are not only fuelled by surface input but rather by chemolithoautotrophic primary production for which we found a high genetic potential (Herrmann *et al.*, 2020; **Chapter 6**) as wells as efficient recycling of bacterial necromass by heterotrophic bacteria and bacterivorous protozoa (Geesink *et al.*, in preparation; **Chapter 5**).

Necromass. Besides in-situ produced biomass through chemolithoautotrophic primary production bacterial communities can recycle dead bacterial biomass. Especially in environments with limited inputs of organic carbon, an efficient use of all available carbon sources is crucial. The ability to recycle necromass has previously been shown for microbial communities in different environments, including marine sediments, soils and compost (Bradley *et al.*, 2018; Müller *et al.*, 2018; Hanajima *et al.*, 2019; Liang *et al.*, 2019) Equivalent to the microbial loop in aquatic systems (Azam *et al.*, 1983), the existence of a "subsurface microbial loop" has been postulated (Dong *et al.*, 2018). By following the uptake of dead bacterial biomass by the microbial community, we were able to demonstrate the potential for recycling of necromass by groundwater microorganisms. Here, necromass mainly serves as a carbon source for heterotrophic organisms belonging to the genera *Flavobacterium, Massilia, Rheinheimera, Rhodoferax* and *Unibacterium* (Geesink *et al*., in prep.; **Chapter 5**).

While heterotrophic organisms directly benefit from the uptake of necromass-derived carbon auto- or mixotrophic members of the community like *Nitrosomonas, Acidovorax*, *Limnohabitans* and *Paucibacter* subsequently benefit from an increasing amount of nitrogen

and sulfur compounds that are released during the metabolization of amino acids by heterotrophs (Geesink *et al.*, in prep.; **Chapter 5**). Thus, also autotrophic primary production is supported by the recycling of dead microbial biomass by the groundwater community.

Additionally, necromass, mainly consisting of proteins, is a source of amino acids, but also lipids or vitamins for heterotrophic bacteria, including metabolically restricted members of the CPR that comprise a major fraction of groundwater communities (Brown *et al.*, 2015; Luef *et al.*, 2015; Geesink *et al.*, 2019; **Chapter 4**; Herrmann *et al.*, 2019; **Chapter 3**). Members of the CPR have indeed been proposed to be involved in the degradation of necromass (Orsi *et al.*, 2018; Geesink *et al.*, 2019; **Chapter 4;** Starr *et al*., 2018). However only one OTU of *Cand.* Gracilibacteria incorporate necromass-derived ^{13}C in our experiments. Indeed, the dependency of *Cand.* Gracilibacteria on externally derived amino acids has recently been proposed (Sieber *et al.*, 2019). The amount of necromass present in the groundwater is supposedly lower than in our microcosm experiment and might have instead over-stimulated growth of more competitive members of the groundwater microbiome. While we were not able to demonstrate the metabolization of necromass-derived carbon multiple members of the CPR, it cannot be excluded to be a relevant process within the groundwater ecosystem.

The OC provided by dead microbial biomass does not only serve as a carbon source for the prokaryotic community, but it is also incorporated by eukaryotes inhabiting the groundwater. Protozoa in groundwater are mainly bacterivorous and thus, take up biomass-derived carbon when feeding on bacteria (Geesink *et al.*, in prep.; **Chapter 5**). At the same time, dissolved organic matter, putatively including necromass, can form larger aggregates that are taken up by the protozoan community (Kerner *et al.*, 2003). Yet, a differentiation between the direct uptake by the labelled necromass or the transfer of carbon through heterotrophic bacteria was not possible (Geesink *et al.*, in prep.; **Chapter 5**). While in marine sediments, the rates of cell death can only cover the maintenance costs of non-growing microorganisms (Bradley *et al.*, 2018), groundwater ecosystems like the Hainich CZE might benefit from the inputs of microorganisms from surface environments (Herrmann *et al.,* 2019; **Chapter 3**). An efficient recycling of the introduced microbial biomass supports heterotrophic and subsequently autotrophic growth (Geesink *et al.,* in prep.; **Chapter 5**). The resulting biomass, combined with a generally increased potential for chemolithoautotrophic primary production putatively supports complex microbial food webs in the subsurface (Herrmann *et al.*, 2020; **Chapter 6**; Geesink *et al.*, in prep.; **Chapter 5**).

Microorganisms inhabiting groundwater have adapted to the conditions within this nutrient-limited environment by being able to temporarily reduce their cell size in phases of starvation and at the same time can rapidly respond to available sources of organic carbon (Herrmann *et al.*, 2019; **Chapter 3;** Geesink *et al.*, in prep.; **Chapter 5**).

Collectively the findings presented within this thesis confirm the third hypothesis:

Groundwater microorganisms respond to the availability of different carbon sources

Within three independent studies we were able to show how available organic carbon rapidly leads to responses in different groups of the microbial community. Organic carbon starvation leads to an immediate decrease in the cell size of the majority of heterotrophic bacteria. At the same time, carbon derived from necromass is rapidly metabolized by heterotrophic community members and subsequently promotes the growth of autotrophic bacteria as well as protozoa. Similarly, organic carbon provided by chemolithoautotrophic primary production supports complex food web structures within the eukaryotic community.

7.3 Advantages of Comprehensive Approaches in Environmental Microbial Ecology

While cultivation-based methods to asses microbial diversity have been shown to only give limited information about the entire diversity of life in an environment, sequencing-based techniques generate a far more complete picture of the bacterial diversity in groundwater (**Figure 6**). Although sequencing of 16S rRNA gene amplicons showed that bacteria belonging to the CPR dominate the groundwater of the Hainich CZE (Herrmann *et al.*, 2019; **Chapter 2**), certain groups of CPR, like *Cand.* Roizmanbacteria, are being overlooked by these methods and could only be detected by genomic-based methods (Geesink *et al.*, 2019; **Chapter 4**). *Cand.* Roizmanbacterium ADI133 was discovered using a cell-sorting based approach and has subsequently been shown to be an abundant member of the groundwater community (Geesink *et al.*, 2019; **Chapter 4**). Due to insertions in their 16S rRNA gene, members of the CPR often show high amounts of mismatches against commonly used primer sets (Brown *et al.*, 2015; Eloe-Fadrosh *et al.*, 2016) and thus could not be detected in the amplicon-based monitoring of the groundwater community (Geesink *et al.*, 2019; **Chapter 4**). Similar effects have been observed for certain protozoan groups that remain undetected by 18S rRNA gene primer sets (Glaser *et al.*, 2014; Johnke and Chatzinotas, 2015). The development of specific primer sets to follow abundances of specific groups can be a time- and cost-effective tool to monitor organisms in complex environments (Geesink *et al.*, 2019; **Chapter 4**).

Besides giving a more complete picture of the diversity within a sample, genome-resolved metagenomic techniques as well as single cell genomics do also improve our understanding on the metabolic potential of an organism in its environment (Rinke *et al.*, 2014; Anantharaman *et al.*, 2016). By that, we can draw conclusion about the potential ecological role and function of yet uncultivated bacteria in the environment (Geesink *et al.*, 2019; **Chapter 4**). In turn, genome-resolved studies can guide and improve cultivation efforts. Examples of the successful use of genome-derived information to cultivate as-yet uncultivated microorganisms include for example *Leptospirillum ferrodiazotrophum* (Tyson *et al.*, 2005), *Pelagibacter ubique* (Carini *et al.*, 2013) and more (reviewed in Gutleben *et al.*, 2018). However, tayloring cultivation approaches towards specific organisms within complex communities remains difficult. The development of new cultivation approaches like “reverse genomics isolation” (Cross *et al.*, 2019) is needed to specifically target individual members of microbial. Cross and colleagues (2019) make use of antibodies against surface proteins of *Saccharibacteria* (TM7) that were predicted based on MAGs or SAGs. The antibodies can be employed to stain and subsequently isolate the target-organisms using cell-sorting techniques (Cross *et al.*, 2019). This technique

offers the possibility for a targeted sorting of yet uncultivated bacteria, including host-associated organisms, from complex communities like the groundwater microbiome.

At the same time, having the genetic potential to perform certain processes in an environment, does not mean, that an organism is indeed making use of these genes. By combining these methods with stable isotope probing (SIP) approaches, not only the expression of genes or the production of certain proteins by an organism can be shown, but also the active incorporation of labelled substrates into an organism can be proven (Jehmlich *et al.*, 2010). With that, Protein-SIP can be used to verify or falsify hypotheses that were made based on prediction from genomes. In turn, these results can be used to revise cultivation efforts as demonstrated by Belnap *et al.* (2010), who used proteomics to verify if their used cultivation conditions for microbial communities resembled the natural communities on a functional level. While the proposed uptake of necromass by *Cand.* Roizmanbacterium ADI133 (Geesink *et al.*, 2019; **Chapter 4)** could not be demonstrated within the framework of this dissertation, the recycling of necromass was demonstrated to be a potential key mechanism for sustaining not only heterotrophic bacteria but a substantial part of the groundwater microbiome via carbon flow to higher trophic levels and release of inorganic electron donors for chemolithoautotrophic metabolisms (Geesink *et al.*, in prep.; **Chapter 5**).

7.4 Conclusion

Within the framework of this dissertation, a holistic approach was applied to elucidate the complex interactions and metabolic potential of groundwater microorganisms in the Hainich Critical Zone Exploratory (**Figure 10**). To address the proposed hypotheses, cultivation-dependent and -independent microbial ecological methods were combined with experimental approaches, to obtain an as complete picture of groundwater community as possible. The findings obtained within this thesis highlight the importance of combining different methods to assess the potential of microbial interactions in complex communities. At the same time, also the limitations of deducing the role of an organisms within the environment based on genomic information are demonstrated with this work. While all methods used within this thesis have different limitations regarding their use in microbial ecology, this dissertation shows how the combination of different methods facilitates a more complete picture of the complexity and potential of microbial communities. The presented findings illustrate how groundwater microbial communities are structured and influenced by species interactions as well as by the metabolic potential of individual members. This thesis further highlights the importance of

trophic interactions in the terrestrial subsurface and the implications that these interactions can have on an entire microbiome. At the same time, cultivation should still be regarded as the most important tool to fully understand the metabolic potential of individual organisms and thus, also the interactions between members of the microbial community. Therefore, the knowledge gained by multi*omics approaches should be used to improve cultivation strategies of yet uncultivated microorganisms, including members of the CPR.

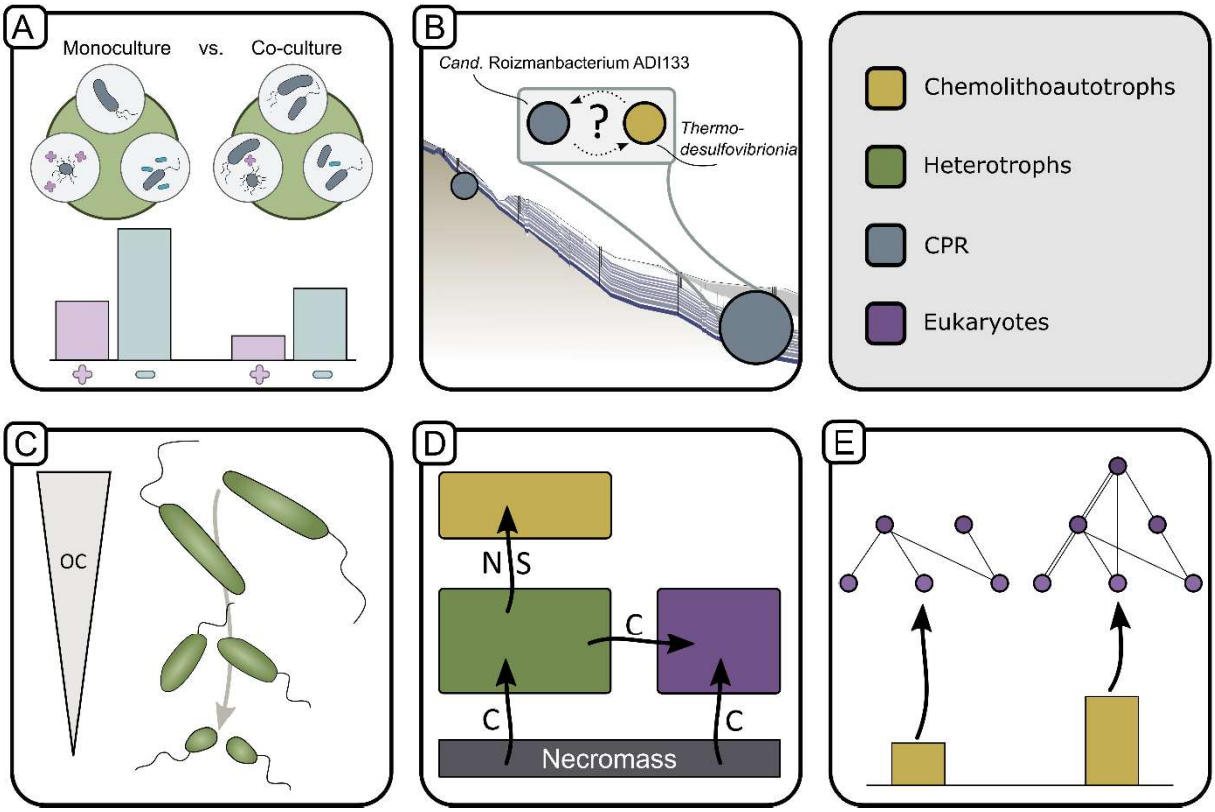

Figure 10 | Forms of interactions and metabolic potential of groundwater microorganisms.
(A) The production of growth-promoting (+) and growth-inhibiting (-) secondary metabolites by groundwater bacteria decreases during co-cultivation (Geesink *et al.*, 2018; Chapter 2). (B) The analysis of the genome of *Cand.* Roizmanbacterium ADI133 elucidated its ecological preferences and potential relationship with an autotrophic partner (Geesink *et al.*, 2019; Chapter 4). (C) Heterotrophic bacteria adjust their cell size to different concentrations of organic carbon (OC) (Herrmann *et al.*, 2019; Chapter 3). (D) Carbon (C) derived from Necromass is incorporated by heterotrophic bacteria and eukaryotes. Nitrogen (N) and sulfur (S) compound released through amino acid degradation support the growth of autotrophic bacteria (Geesink *et al.*, in prep.; Chapter 5). (E) An increasing genetic potential for chemolithoautotrophy putatively supports complex food webs in groundwater (Herrmann *et al.*, 2020; Chapter 6).

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Declaration of Authorship

I hereby affirm that I composed this dissertation by myself and only with the use of resources, data, personal communications, and literature cited in the text. Those who provided assistance for the experiments, data analyses, or writing of the manuscripts are listed as co-authors or mentioned in the acknowledgements in the respective chapters.

Furthermore, I confirm that I have read and fully understood the 'Course of Examination for Doctoral Candidates' (Promotionsordnung, September 23rd, 2019) by the Faculty of Biological Sciences of the Friedrich Schiller University Jena.

I did not obtain any assistance from a consultant for doctorate theses, and no third parties have received any indirect or direct financial rewards in relation with the contents of this dissertation. This dissertation or parts of it have not been previously submitted as thesis for scientific survey to the Friedrich Schiller University Jena or to any other university.

Jena, 19.12.2019

P. Geesink

Patricia Geesink

Published Articles and Pending Manuscripts

The thesis chapters 2, 3, 4 and 6 were published in international, peer reviewed journals. Chapter 5 is a manuscript draft in preparation. My contribution to each manuscript were:

Chapter 2 **Growth promotion and inhibition induced by interactions of groundwater bacteria**
(Geesink *et al.*, 2018; published in FEMS Microbiology Ecology)

For this study, I designed the experiment together with KK and PG and developed the screening method in collaboration with PG and OT. I isolated the majority of the studied bacteria with contributions from MT and SK and performed the screening with help from CV. I evaluated, analysed and interpreted the data together with MT and OT. The manuscript was written by me with contributions from all authors. My contribution can be summarized as following:

Study Design:	60%
Experimental Work:	90%
Data Analysis:	80%
Manuscript Writing:	70%

Chapter 3 **Predominance of *Cand.* Patescibacteria in groundwater is caused by their preferential mobilization from soils and flourishing under oligotrophic conditions**
(Herrmann *et al.*, 2019; published in Frontiers in Microbiology)

I performed and analysed the bacterial starvation experiment and, together with MH, contributed to the molecular work for this study. I was involved in the data interpretation, discussions and the preparation of figures for this manuscript. MT, MH and KK wrote the manuscript with contributions from all authors. My contribution can be summarized as following:

Study Design:	5%
Molecular Work:	20%
Experimental Work:	70%
Data Analysis:	10%
Manuscript Writing:	5%

Chapter 4 **Genome-inferred spatio-temporal resolution of an uncultivated Roizmanbacterium reveals its ecological preferences in oligotrophic groundwater**
(Geesink *et al.*, 2019; published in Environmental Microbiology)

This study was designed by me AJP and KK. The Single Cell Sorting was performed by MH, HT and AKK. I subsequently screened the Minimetagenomes and prepared the samples for sequencing. Together with AJP and CEW I reconstructed and analysed the genome of *Cand.* Roizmanbacterium ADI133. I developed the qPCR assay for Roizmanbacteria.

Together with MH I performed the co-occurrence network analysis. The manuscript was written by me with contributions from all authors. My contribution can be summarized as following:

Study Design:	70%
Molecular Work:	90%
Data Analysis:	80%
Manuscript Writing:	80%

Chapter 5 **Bacterial necromass is rapidly metabolized by heterotrophic members of the microbial community in groundwater**

(Geesink *et al.*, manuscript in preparation)

This study was designed by me, MT and KK. I performed the experimental and molecular work for this study together with MT. The proteomic analyses were performed in a collaboration with NJ and MB. Together with MT I analysed the data obtained from the DNA- and Protein-SIP experiment and wrote the manuscript draft with contributions from MT. My contribution can be summarized as following:

Study Design:	70%
Molecular Work:	100%
Experimental Work:	80%
Data Analysis:	70%
Manuscript Writing:	80%

Chapter 6 **Complex food webs coincide with high genetic potential for chemolithoautotrophy in fractured bedrock groundwater**

(Herrmann *et al.*, 2020; published in Water Research)

Together with MH, I contributed to the molecular work including RNA extractions and quantitative PCRs for this manuscript. I was involved in the data interpretation, discussions and the preparation of figures for the manuscript. Furthermore, I reviewed and revised versions of the manuscript. The manuscript was written by MH with contributions from all authors. My contribution can be summarized as following:

Study Design:	0%
Molecular Work:	20%
Data Analysis:	10%
Manuscript Writing:	5%

Jena, _____

Patricia Geesink

Further Articles

The following manuscript is currently submitted for publication to *Environmental Microbiology* and can be found as a pre-print on bioRxiv.org

Inclusion of Oxford Nanopore long reads improves all microbial and phage metagenome-assembled genomes from a complex aquifer system

Will A. Overholt, Martin Hölzer, **Patricia Geesink**, Celia Diezel, Manja Marz, Kirsten Küsel

published online on December 19th, 2019 on bioRxiv.

Assembling microbial and phage genomes from metagenomes is a powerful and appealing method to understand structure-function relationships in complex environments. In order to compare the recovery of genomes from microorganisms and their phages from groundwater, we generated shotgun metagenomes with Illumina sequencing accompanied by long reads derived from the Oxford Nanopore sequencing platform. Assembly and metagenome-assembled genome (MAG) metrics for both microbes and viruses were determined from Illumina-only assemblies and a hybrid assembly approach. Strikingly, the hybrid approach more than doubled the number of mid to high-quality MAGs (> 50% completion, < 10% redundancy), generated nearly four-fold more phage genomes, and improved all associated genome metrics relative to the Illumina only method. The hybrid assemblies yielded MAGs that were on average 7.8% more complete, with 133 fewer contigs and a 14 kbp greater N50. Furthermore, the longer contigs from the hybrid approach generated microbial MAGs that had a higher proportion of rRNA genes. We demonstrate this usefulness by linking microbial MAGs containing 16S rRNA genes with extensive amplicon dataset. This work provides quantitative data to inform a cost-benefit analysis on the decision to supplement shotgun metagenomic projects with long reads towards the goal of recovering genomes from environmentally abundant groups.

Curriculum Vitae

Patricia Geesink, M.Sc.
[redacted]

EDUCATION

[redacted]

ACADEMIC POSITIONS

[redacted]

TEACHING AND SUPERVISION EXPERIENCE

[redacted]

AWARDS AND GRANTS

[redacted]

PUBLICATIONS

Risse-Buhl, U., Herrmann, M., **Lange, P**., Akob, D.M., Pizani, N., Schönborn, W., et al. (2013) Phagotrophic Protist Diversity in the Groundwater of a Karstified Aquifer - Morphological and Molecular Analysis. *J. Eukaryot. Microbiol.* **60**: 467–479.

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Taubert, M., Stöckel, S., **Geesink, P**., Girnus, S., Jehmlich, N., von Bergen, M., et al. (2018) Tracking active groundwater microbes with D2O labelling to understand their ecosystem function. *Environ. Microbiol.* **20**: 369–384.

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Geesink, P., Wegner, C., Probst, A.J., Herrmann, M., Dam, H.T., Kaster, A., and Küsel, K. (2019) Genome-inferred spatio-temporal resolution of an uncultivated Roizmanbacterium reveals its ecological preferences in groundwater. *Environ. Microbiol.* 1462-2920.14865.

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CONFERENCE CONTRIBUTIONS

2015

Cassandre S. Lazar, Martina Herrmann, **Patricia Lange**, Kai Uwe Totsche, Kirsten Küsel (2015) Ammonia-oxidizing Thaumarchaeota form a large fraction of the archaeal community in pristine limestone aquifers. Goldschmidt Conference (Prague; Czech Republic), poster presentation.

Swatantar Kumar, Martina Herrmann, **Patricia Lange**, Valerie Schwab-Lavric, Kai Uwe Totsche, Susan Trumbore, Kirsten Küsel (2015) Abundance and community composition of microbial groups involved in denitrification and anammox in two superimposed limestone aquifers differing in oxygen availability. VAAM (Marburg, Germany), poster presentation.

2016

Martina Herrmann; Lena Carstens, **Patricia Lange**, Michael Gaspar, Kai Uwe Totsche, Kirsten Küsel (2016) Abundance and community structure of groundwater microorganisms that pass through 0.2 µm pore size filters. VAAM (Jena, Germany), poster presentation.

Swatantar Kumar, Martina Herrmann, **Patricia Lange**, Kai Uwe Totsche, Susan Trumbore, Kirsten Küsel (2016) Diversity and distribution of anammox bacteria and denitrifiers in pristine limestone aquifers. VAAM (Jena, Germany), poster presentation.

Martina Herrmann, **Patricia Lange**, Martin Taubert, Lena Carstens, Christine Hess, Cassandre S. Lazar, Kai Uwe Totsche, Kirsten Küsel (2016) Be small and find a host – do oligotrophic subsurface environments support symbiotic and parasitic relationships among bacteria? iDiv Conference 2016 (Leipzig, Germany), oral presentation.

Patricia Lange, Martina Herrmann, Lena Carstens, Christine Steinhäuser, Michael Gaspar, Kirsten Küsel (2016) Size matters – Impact of organic carbon availability on cell size of groundwater microbial communities. ISME16 (Montreal, Canada), poster presentation.

Martina Herrmann, Valérie F. Schwab, Martin Nowak, Swatantar Kumar, Cassandre S. Lazar, **Patricia Lange**, Susan Trumbore, Gerd Gleixner, Kai-Uwe Totsche, Kirsten Küsel (2016) High spatial heterogeneity of organic carbon input and autotrophic potential in pristine limestone aquifers; ISME16 (Montreal, Canada), poster presentation.

Swatantar Kumar, Martina Herrmann, Valerie Schwab-Lavric, **Patricia Lange**, Bo Thamdrup, Kai Uwe Totsche, Susan Trumbore, Kirsten Küsel (2016) Contribution of anammox versus denitrification to nitrate removal in pristine aquifers. ISME16 (Montreal, Canada), poster presentation.

2017

Patricia Geesink, Olaf Tyc, Kirsten Küsel, Paolina Garbeva (2017) Love and Hate in the Subsurface - Growth Promotion and Inhibition induced by Groundwater Bacteria. MiCom (Jena, Germany), oral presentation.

Martina Herrmann, Swatantar Kumar, **Patricia Geesink**, Bo Thamdrup, Valerie F. Schwab, Sebastian Lücker, Kai Uwe Totsche, Kirsten Küsel (2017) Hotspots of anammox and nitrification in oligotrophic karstic limestone aquifers. ICoN5 (Vienna, Austria), poster presentation.

Martin Taubert, Stephan Stöckel, **Patricia Geesink**, Sophie Friedrich, Nico Jehmlich, Martin von Bergen, Petra Rösch, Jürgen Popp, Kirsten Küsel (2017) Tracking active microbes to understand their ecosystem function. GRC AEM (South Hadley; USA), poster presentation.

Patricia Geesink, Martina Herrmann, Karin Potthast, Tim Richter, Kirsten Küsel, Beate Michalzik (2017) iBeLeaf – Simulated aphid infestation induces changes in phyllosphere microbiome and biogeochemical cycles. iDiv Conference (Leipzig, Germany), oral presentation.

Constantinos Xenophontos, Carl-Eric Wegner, **Patricia Geesink**, Swatantar Kumar, Stanley Harpole, Kirsten Küsel (2017) Shifts in ecological functioning of groundwater bacteria driven by species interactions. iDiv Conference (Leipzig, Germany), poster presentation.

Patricia Geesink, Martina Herrmann, Carl-Eric Wegner, Kai Uwe Totsche, Kirsten Küsel (2017) Being a dwarf versus being starved – ultrasmall bacteria in oligotrophic groundwater. ISSM (Rotorua, New Zealand), oral presentation.

Martina Herrmann, Swatantar Kumar, **Patricia Geesink,** Bo Thamdrup, Valerie F. Schwab, Sebastian Lücker, Kai Uwe Totsche, Kirsten Küsel (2017) Hotspots of anammox and nitrification in oligotrophic karstic limestone aquifers; ISSM (Rotorua, New Zealand), oral presentation.

2018

Patricia Geesink, Lisa Mahler, Carl-Eric Wegner, Martin Roth, Kirsten Küsel (2018) Exploring microbial dark matter with pL droplets. CBM Workshop (Ilmenau, Germany), poster presentation.

Lisa Mahler[1], **Patricia Geesink**[1], Carl-Eric Wegner, Martina Herrmann, Miriam A. Rosenbaum, Martin Roth, Kirsten Küsel (2018) Illuminating microbial dark matter in the macro- and microscale. Life meets Light Conference (Jena, Germany), oral presentation.

Patricia Geesink, Carl-Eric Wegner, Martina Herrmann, Hang Thuy Dam, John Vollmers, Anne-Kristin Kaster, Kirsten Küsel (2018) Unravelling the lifestyle and metabolic potential of *Cand.* Patescibacteria in oligotrophic aquifers. ISME17 (Leipzig, Germany), poster presentation.

Martina Herrmann, **Patricia Geesink**, Kirsten Küsel (2018) The secret life of microbes in hardwood forest canopies. iDiv Conference (Leipzig; Germany), oral presentation.

Martina Herrmann, Carl-Eric Wegner, Swatantar Kumar, **Patricia Geesink**, Bo Thamdrup, Kai Uwe Totsche, Kirsten Küsel (2018) Hotspots of anammox and nitrification in oligotrophic karstic limestone aquifers. VAAM (Wolfsburg, Germany), oral presentation.

[1] Co-presenting authors

2019

Beate Michalzik, Tim Richter, Alexander Tischer, Kirsten Küsel, **Patricia Geesink**, Martina Herrmann (2019) Tree species metrics and canopy microbial communities affect aboveground hydrology and biogeochemistry. EGU (Vienna, Austria), oral presentation.

Martina Herrmann, **Patricia Geesink**, Lijuan Yan, Robert Lehmann, Kai Uwe Totsche, Kirsten Küsel (2019) Complex food webs in groundwater coincide with high genetic potential for chemolithoautotrophy. iDiv Conference (Leipzig, Germany), oral presentation.

Patricia Geesink, Carl-Eric Wegner, Alexander J. Probst, Martina Herrmann, Hang Thuy Dam, John Vollmers, Anne-Kristin Kaster, Kirsten Küsel (2019) Genome-inferred spatio-temporal resolution of an uncultivated Roizmanbacterium reveals its ecological preferences in oligotrophic groundwater. GRS and GRC AEM (South Hadley, USA), poster presentation.

Martina Herrmann, **Patricia Geesink**, Lijuan Yan, Robert Lehmann, Kai Uwe Totsche, Kirsten Küsel (2019) Complex food webs in groundwater coincide with high genetic potential for chemolithoautotrophy. SAME (Potsdam, Germany), oral presentation.

Jena, 19.12.2019

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